Author:
Wareham, Kezia

Title:
Risk factors for the carriage of antimicrobial-resistant Escherichia coli in puppies and adult dogs

Risk factors for the carriage of antimicrobial-resistant *Escherichia coli* in puppies and adult dogs

Submitted by Kezia Wareham

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of MSc by Research in Clinical Veterinary Science from Bristol Veterinary School, Faculty of Health Sciences

October 2018

-

Word Count – 19186

Abstract

Antimicrobial resistance (AMR) is a major worldwide issue with severe implications on human and animal health as well as global food security. For this study, 223 (41 locally recruited and 182 through the Dog's Trust Generation Pup study) 16-week-old puppies, 64 locally recruited 12-week-old puppies and 25 adult dogs (14 locally recruited and 11 recruited from the Oxfordshire area) were screened and their owners provided a faecal sample from their dog and completed a questionnaire. The *E. coli* (*Escherichia coli)* carried by these puppies were tested for resistance to five different antimicrobials (ciprofloxacin, streptomycin, tetracycline, cephalexin and amoxicillin). Univariable and multivariable logistic regression analyses were used to explore possible risk factors for AMR in *E. coli* carried by the puppies. Puppies that were fed raw meat had a greater risk of ciprofloxacin resistance (multivariable: 12.42 (5.01 to 30.78) <0.001), tetracycline resistance (multivariable: 4.47 (2.21 to 9.05) <0.001), amoxicillin resistance (multivariable: 3.18 (1.57 to 6.42) 0.001) and streptomycin resistance (multivariable: 8.23 (3.95 to 17.15) <0.001). Autocoprophagia was found to be protective against resistance in the Generation Pup cohort to tetracycline (multivariable: 0.10 (0.01 to 0.80) 0.03) and amoxicillin (multivariable: 0.18 (0.04 to 0.82) 0.03). The cephalexin resistant *E. coli* were screened for cefotaxime resistance and further molecular methods were carried out on these. The PCR and whole genome sequence results from the cefotaxime- and ciprofloxacin-resistant *E. coli* carried by the puppies showed that there were a variety of sequence types (ST's) identified and provided the mechanism for resistance. For the local cohort of puppies' samples were collected at 12 and 16 weeks to allow for comparison of resistance. Amoxicillin resistance in the *E. coli* isolated from the 12-week-old puppies was higher in comparison to the 16-week-old puppies ($p<0.001$). There was evidence of regional variation in resistance depending on the recruitment area. Tetracycline ($p=0.05$) and amoxicillin ($p=0.04$) resistance were more common in *E. coli* isolated from the 16-week-old puppies that were locally recruited compared to those that were recruited nationally. Furthermore, adult dogs recruited from the Oxfordshire area (n=11) were more likely to have amoxicillin resistance compared to locally recruited dogs (n=14; $p=0.02$).

Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the *University's Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: [redacted] DATE: 01/10/18

Acknowledgments

I would firstly like to thank everyone who assisted me throughout my MSc by research. I would like to thank my supervisors Dr. Kristen Reyher and Dr. Tristan Cogan for all of their support, help and encouragement and with thanks to Professor Matthew Avison for his help and sharing of his expertise.

I would also like to thank the OH-STAR team including Dr. Jacqueline Findlay and Oliver Mounsey for providing me with the PCR and whole genome sequence data as well as Dr. Ginny Gould for her help and support with recruitment of the puppies in this study. I am grateful to Dr. Ashley Hammond for providing me with the models for the risk factor analysis. I also wish to thank Generation Pup for help with recruitment for this study and for providing data for the puppies.

Finally, I would like to thank all those who helped with recruitment for this study and I would like to show my appreciation for all of the puppy and dog owners who took part in my research study.

Table of Contents

Table of Tables and Figures

List of Abbreviations

AMR	Antimicrobial resistance
BL	Beta-lactamase
BSAVA	British small animal veterinary association
Cfu	Colony-forming-unit
E. coli	*Escherichia coli*
ESBL	Extended spectrum β-lactamase
GDP	Gross domestic product
MLST	Multilocus sequence typing
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RAPD	Random Amplification of Polymorphic DNA
RUMA	Responsible use of medicines in agriculture
ST's	Sequence Types
TBX agar	Tryptone Bile X-Glucuronic agar
WHO	World Health Organisation

Chapter 1 – Introduction

1.1 Antimicrobial Resistance (AMR)

Antimicrobial resistance (AMR) is a major worldwide issue with serious consequences and has been described as 'one of the greatest health threats faced today' (Davies *et al.* 2011). AMR has many negative impacts on the health and welfare of humans and animals including increased morbidity and mortality as well as prolonged illness and may result in treatments being expensive, difficult or ineffective (Friedman *et al.* 2015*).* The problems are so severe that the United Nations General Assembly along with world leaders in the G7 and the G20 have stated that AMR is a global crisis (Bloom *et al.* 2017). The 21st century has been described as a post-antibiotic era in which common infections could cause death and a global action plan of interventions is needed to mitigate AMR (WHO report, 2014). The magnitude of the problem is still not entirely known and therefore further research is required into the complex challenge. If current trends continue, the rates of morbidity and mortality from infections caused by AMR bacteria will increase (deKraker *et al.* 2016). Understanding the threat of AMR will not only help preserve antimicrobials but will also enable the development of new antimicrobials or alternative approaches to control infections (Prescott & Boerlin, 2016).

The problem associated with AMR are that resistance to new antibiotics arises sooner or later and there is a lack of development of new antimicrobials (Ferri *et al.* 2015). The current dearth of new antimicrobial drug development by the pharmaceutical industry can be attributed to reduced economic incentives and challenging regulations and polices (Ventola *et al.* 2015). Furthermore, the lack of research and development of novel antimicrobials could be due to a certain amount of discouragement due to the fact that new molecules may quickly become resistant and therefore ineffective in a short period and that it is not always financially viable to develop new antimicrobials (Ferri *et al.* 2015). Antimicrobials have been used to treat infections for decades but have been misused in human health care as well as in veterinary care, causing them to

become increasingly ineffective. AMR will have serious consequences and it has been estimated that by 2050, 10 million people will die every year due to AMR unless the issue is taken seriously (de Kraker *et al.* 2016).

As well as having severe impacts on human and animal health, AMR also has an economic burden and has led to increasing costs in healthcare and could even potentially lead to the destabilisation of health systems (Ferri *et al.* 2015). Health care costs are often increased for patients suffering with AMR infections as a longer recovery may be required and more expensive drugs may need to be used (Ferri *et al.* 2015). It has been estimated that there will be a reduction of 3% in the world gross domestic product (GDP) by 2050 if an effective global AMR strategy is not implemented (Sirijatuphat *et al.* 2017).

AMR will also have an impact on the livestock industry as it may become more difficult to treat resistant infections in livestock, which could create an economic burden on livestock producers and may increase prices for consumers. In veterinary medicine, antimicrobials are crucial to maintain animal health, animal welfare and food safety (Magouras *et al.* 2017). AMR is linked between human populations, animal populations and the environment, and it is possible for resistance to be passed between these populations (Woolhouse *et al.* 2015). To enable a better understanding of the role antimicrobials play in human and veterinary medicine it is important to monitor antimicrobial usage in food-producing animals (Hockenhull *et al.* 2017). Widespread antimicrobial usage in agriculture may contribute to the development of resistance in humans especially as there is an overlap of antibiotics used in humans and in food-producing animals (Tang *et al.* 2017). Research has suggested that bacteria in animals that are treated with antibiotics can develop resistance and that bacteria carrying resistance genes can be transmitted from animals to humans (Tang *et al.* 2017; Liu *et al.* 2018). Some research has indicated that food-producing animals act as reservoirs of resistance genes which could potentially be transmitted to humans through the food chain, via direct contact or through the environment, however the extent of this transmission is currently unknown (Zurfluh *et al.* 2015; Magouras *et al.* 2017). Antimicrobials have been inappropriately used in the livestock industry; for example, in the pig industry antimicrobials were used for the treatment and prevention of disease as well as for other benefits such as

growth promotion and feed conversion efficiency, however, attempts have been made to limit the inappropriate use of antimicrobials in the livestock industry (Stevens *et al.* 2007). Strategies need to be developed to reduce AMR in humans and animals and these approaches need to be coordinated at national and international levels. The evidence suggests that there isn't a single solution to this problem and therefore several approaches need to be taken synergistically (Holmes *et al.* 2016*).*

Antimicrobials, however, are vital to treat infections in companion animals, livestock animals and humans. It is therefore important to fully understand potential risk factors associated with transmission of AMR in an attempt to identify ways to reduce its severity for farming, global food security, animal welfare and both human and veterinary medicine.

1.2 Development of Antimicrobial Resistance

AMR is believed to be a natural phenomenon that is accelerated by selection pressure from the use and misuse of antimicrobials in humans and animals (WHO, 2014; Wedley *et al.* 2011). Multidrug resistance is when a bacterium has resistance to three or more classes of antimicrobials (Wedley *et al.* 2017). The lack of new antimicrobials to replace ineffective ones means that the existing drugs need to be protected (WHO 2014). Resistance can be acquired via mutation of chromosomal DNA or by horizontal transfer of resistance via transmissible elements called plasmids (Wedley *et al.* 2011). Bacteria are able to respond to the environmental threat of antimicrobial molecules with two genetic strategies which both result in resistance: mutations in the genes and horizontal gene transfer (Munita & Arias 2016).

1.2.1. Mutational Resistance

Bacteria can acquire resistance to an antimicrobial through mutation and a Darwinian selection process which arose to evade destruction from many toxic

substances (Holmes *et al.* 2016). Resistance can arise when an antimicrobial attacks a bacterial cell; those that are susceptible die, however those that do not succumb survive and replicate in the presence of the antimicrobial and the emergence of this resistant phenotype will spread (Giedraitienė *et al.* 2011; Munita & Arias 2016). Most antimicrobials are naturally derived from microorganisms such as environmental fungi and saprophytic bacteria or are synthetic modifications of these; only a small number are wholly synthetic and therefore bacteria have evolved mechanisms to protect themselves from antimicrobials (Holmes *et al.* 2016). Once there is a mutation that makes the bacteria resistant and once under selective pressure, the susceptible population without the mutation are killed and the bacteria that are newly resistant survive and grow (Tenover 2006; Munita & Arias 2016). There are a number of different ways that mutations may cause resistance, this could be by altering the target protein the antimicrobial molecule binds to, by removing or modifying the binding site or by upregulating the production of enzymes that inactivate the antimicrobial as well as a number of other ways (Tenover 2006).

1.2.2. Horizontal gene transfer

The second type of antimicrobial resistance is horizontal gene transfer which is the acquisition of new genetic material from resistant organisms or the transfer of resistance genes from one bacterium to another (Giedraitienė *et al.* 2011; Tenover 2006; Munita & Arias 2016). Horizontal gene transfer is the acquisition of genes from another source (usually bacteria) and can be between the same species or between different species of bacteria (Tenover 2006; Munita & Arias 2016).

There are three methods for bacteria to acquire external genetic material: via transformation, transduction or conjugation (Bennett *et al.* 2008). During transformation the bacterial cell takes up ‘naked’ DNA (which could have resistance genes) from its environment and integrates the DNA into its own genome (Bennett *et al.* 2004). Bacterial transformation is usually from the same or closely related species due to self-DNA recognition (Bennett *et al.* 2004). Resistance genes can also be transferred by bacterial viruses (bacteriophages)

during transduction (Bennett *et al.* 2004). Conjugation requires mobile genetic elements such as plasmids and transposons which encode a 'DNA transfer system that has evolved specifically to mediate horizontal transfer of itself' (Munita & Arias 2016; Bennett *et al.* 2004). Plasmids are circular, double-stranded DNA molecules and generally exist separately and independently of the main bacterial chromosome. They do not usually carry core genes needed for basic cell growth and multiplication but instead carry accessory genes that may be useful periodically to enable bacteria to exploit a particular environmental situation, for example, surviving in the presence of potentially lethal antimicrobials (Bennett *et al.* 2008). Resistance plasmids carry a variety of different genes (e.g. those that confer antibiotic resistance and resistance to some toxic heavy metals). A resistance plasmid is any plasmid that carries one or more AMR genes (Bennett *et al.* 2008). Plasmids have been shown to encode genes that confer resistance to different classes of antimicrobials including cephalosporins, fluoroquinolones and aminoglycosides (Giedraitienė *et al.* 2011). Resistance transposons are mobile genetic elements that can change their position within a genome. These can incorporate a resistance gene within the element giving the resistance gene the ability to move within a DNA molecule or from one DNA molecule to another (e.g. from one plasmid to another; Bennett *et al.* 2008).

There are different types of biochemical mechanisms that bacteria use to defend against antimicrobial molecules these include; decreased uptake, enzymatic modification and degradation, altered binding proteins, increased efflux, altered target sites and the overproduction of enzymes (Giedraitienė *et al.* 2011). β-lactamases are enzymes produced by Gram negative bacteria and are coded on chromosomes and plasmids and they are able to hydrolyse many β-lactam antimicrobials (e.g. cephalosporins; Giedraitienė *et al.* 2011).

Bacteria producing extended-spectrum-β-lactamase (ESBL) enzymes are able to survive in the presence of various β-lactam antibiotics including a wide range of clinically useful medicines (penicillins, cephalosporins, etc.; Leonard *et al.* 2017, Giedraitienė *et al.* 2011). Plasmids carrying ESBLs can move between

bacteria and often encode multidrug resistance to antimicrobials including quinolones, aminoglycosides and tetracyclines (Leonard *et al.* 2017). ESBL-producing *Enterobacteriaceae* have been identified by the World Health Organisation (WHO) as being of 'critical priority' for research and for the development of new antibiotics active against this group of bacteria (WHO, 2017). Resistance can be mediated by the acquisition of plasmid-mediated ESBLs which hydrolyse and inactivate antimicrobials. Some *E. coli* strains develop resistance to third generation cephalosporins through the acquisition of ESBLs through mutations of TEM-, SHV- or CTX-M type enzymes (Tenover 2006; Zurfluh *et al.* 2015). Mobile β-lactamase enzymes are given a three letter name (e.g. TEM). In 1988 a publication detailed the detection of ESBL genes in isolates obtained from pet dogs and other studies have reported the presence of the *bla* genes in isolates obtained dogs, especially those from a clinical origin (Carvalho *et al.* 2016).

CTX-Ms are β-lactamase enzymes that belong to a class of ESBLs. The spread of these enzymes has been referred to as the 'CTX-M pandemic' because of the increasing penetrance of these β-lactamase genes (*bla)* across the world (Cantón *et al.* 2012). The origin of CTX-Ms was mobilisation of chromosomal $bla_{\text{CTX-M}}$ genes from *Kluyvera* species into mobile genetic elements such as transposons and plasmids (Zurfluh *et al.* 2015; Cantón *et al.* 2012). CTX-M enzymes can be classified by amino acid similarities into five main groups (members of the same group share over 94% identity): CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (Bonnet, 2004). CTX-M genes are found in *E. coli* in the environment as well as many different animals including humans, dogs, chickens and other animals. CTX-M-15 is described as 'by far one of the most important' CTX-M enzymes as it nearly invades all human and animal compartments as well as environments all over the world and has been estimated to be present in 4% of total ESBL-producing *E. coli* (Cantón *et al.* 2012). CTX-M-1 is the most common ESBL type found in livestock and the second most frequent variant associated with human clinical isolates in some countries including France and Italy (Kjeldsen *et al.* 2015).

TEM-type ESBLs were first reported from an *E. coli* isolate in 1965 and are capable of hydrolysing penicillins and first generation cephalosporins (Shaik *et*

al. 2014). Since 2002, the *bla*$_{TEM}$ has been detected clinically obtained *E. coli* isolated from dogs (Carvalho *et al.* 2016).

OXA-type β-lactamases are named because of their oxacillin-hydrolysing abilities and are found in many Gram negative bacteria (Shaik *et al.* 2014). The most common type is OXA-1 which is reported to have been found in 1-10% of clinically obtained human *E. coli* isolates (Shaik *et al.* 2014).

AmpC β-lactamase is a bacterial enzyme that is able to destroy antimicrobials such as penicillin and is a major clinical concern (Jacoby 2009). Resistance through the overexpression of this enzyme in Gram negative bacteria occurs usually because of deregulation of the *ampC* chromosomal gene or by acquisition of a mobile genetic element (i.e. plasmid) with a transferable *ampC* gene. This is commonly referred to as a plasmid-mediated AmpC β-lactamase (Pérez-Pérez *et al.* 2002).

The emergence of ESBLs and AmpC-producing *E. coli* are particularly concerning as these bacteria are resistant to a variety of β-lactam antimicrobials including highest priority critically important antimicrobials such as third generation cephalosporins (Schmidt *et al.* 2015). ESBL and AmpC-producing *E. coli* have been found worldwide in isolates obtained from humans, food-producing animals, companion animals and the environment (Hordijk *et al.* 2013). ESBL and AmpC-producing *E. coli* have been found in both healthy and ill dogs and an association has been found between the use of antimicrobials in dogs and veterinary healthcare with increased detection of AMR in dogs (Schmidt *et al.* 2015; Damborg *et al.* 2009).

CMY enzymes are plasmid-mediated β-lactamases found in Gram negative bacteria worldwide and can confer carbapenem resistance (Pavez *et al.* 2008). They are thought to have descended from chromosomal *ampC* genes from *Citrobacter freundii* and *Aeromonas spp.* (Naseer *et al.* 2009). CARB-type enzymes are carbenicillin-hydrolysing β-lactamases (Chiou *et al.* 2015).

1.3 *E. coli*

Escherichia coli (E. coli) is a Gram negative bacillus of the *Enterobacteriacae* family. *E. coli* is a facultative aerobe and is commonly found in the gut of animals making it a useful marker for exploring AMR in animals (Blount 2015). *E. coli* can also be easily obtained via faecal samples (Wedley *et al.* 2011). Many *E. coli* found inhabiting the intestines of animals are harmless commensals, however some types cause intestinal and extra-intestinal infections which may require antibiotic treatment (Leonard *et al.* 2017; Timofte *et al.* 2016). *E. coli* are found in many different animal systems and therefore resistance can be compared in different animals; possible transmission between companion animals and humans as well as other animals can also be compared (Murphy *et al.* 2009).

E. coli has become resistant to antimicrobials through a number of mechanisms. These include the overproduction of the target enzyme, modification of antibiotic targets, degradation of the antimicrobial agent as well as other mechanisms (van Hoek *et al.* 2011). *E. coli* can become AMR by chromosomal DNA mutations and also as a result of the acquisition of new genetic material through horizontal gene transfer (van Hoek *et al.* 2011). Through horizontal gene transfer, *E. coli* take up DNA with AMR genes (i.e. plasmids) via transformation, transduction or conjugation (Burmeister 2015).

Resistant *E. coli* is a threat as some *E. coli* may be opportunist pathogens and may be a reservoir of AMR genes for pathogenic or zoonotic bacteria (Murphy *et al.* 2009). *E. coli* is the most frequent cause of community and hospital-acquired urinary tract infections and therefore is a substantial threat to human health (WHO report, 2014).

1.4 Antimicrobial usage in small animals

Evidence suggests that use of antibiotics creates a selection pressure that contributes to increased AMR, therefore it is imperative to investigate the usage

of antibiotics in small animals. In Europe, the prescribing of antimicrobials for use in animals must be done by a veterinarian and the prescription data for antimicrobials is kept at veterinary practice level; as in human medicine, it is therefore very desirable for researchers to collate this data (Radford *et al.* 2011). A study that evaluated data from small animal antimicrobial prescribing patterns in the UK revealed that only a small proportion of investigated veterinary practices followed antimicrobial use guidelines and suggested that the use of guidelines in the UK could reduce antimicrobial use (Hughes *et al.* 2012).

Recent research examining 374 veterinary practices in the UK involving nearly 1 million dogs and 600,000 cats looked at the antimicrobial usage in small animals (Prescott & Boerlin, 2016). It estimated that during the 2 years investigated, approximately 25% of dogs and 21% of cats registered at the practices received at least one course of antibiotics (Prescott & Boerlin, 2016). Of all dog antimicrobial usage, 60% was antimicrobials classified as 'critically important' for human medicine using the WHO criteria (Prescott & Boerlin, 2016). Research has suggested that despite the quantity of antimicrobials for veterinary use sold in the UK decreasing since 2002, the total sales of antimicrobials for non-food-producing animals has increased, especially for usage in dogs (Wedley *et al.* 2011). This increase in use and resultant selection pressures may have a knock-on effect of higher carriage of AMR in small animals (Wedley *et al.* 2011). The high levels of antimicrobial usage in small animals in the UK also indicates that it is important to research AMR in these species and to investigate possible interventions to reduce the impact of AMR. There are concerns that the use of antimicrobials in animals may contribute to resistance in humans which has led to the suggestion that the use of quinolones and cephalosporins should be restricted in animals (Radford *et al.* 2011).

1.5 Dog acquisition of antimicrobial-resistant bacteria

There are a number of ways that dogs may acquire AMR *E. coli.* Some evidence has suggested that animals can acquire resistance from the environment; acquisition through the natural environment has been recognised as a possible

transmission route but has been understudied (Leonard *et al.* 2017). Manure is often used as a fertiliser for crops, and wastewater from run-off which enters waterways may introduce bacteria with diverse mobile genetic elements to coastal waters alongside compounds that select for resistant microorganisms (Leonard *et al* 2017; Amos *et al.* 2014). Studies have shown that ingestion of water containing AMR *E. coli* is associated with gut colonisation by these bacteria (Coleman *et al.* 2012*).* The ingestion of resistant *E. coli* that then colonises the gut of the dog may be a possible route of acquisition of resistant bacteria. Research has also suggested that dogs acquire AMR bacteria when visiting veterinary hospitals which have been shown to act as a reservoir for multi-drug resistant organisms (Gibson *et al.* 2011; Ogeer-Gyles *et al.* 2006; Hutton 2018). The risk of acquiring AMR bacteria from veterinary hospitals could be due to the fact that patients are often susceptible to infection as well as the high selection pressure for resistance due to antibiotic use within practices (Hutton 2018). Studies have concluded that the use of antimicrobial agents selects for and promotes the transfer of AMR. Furthermore, antimicrobials are frequently prescribed to dogs and there is evidence of development of resistance in response to treatment (Singleton *et al.* 2017; Gibson *et al.* 2011; Ogeer-Gyles *et al.* 2006; Trott *et al.* 2004). The close domestic relationship between dogs and their owners has also raised the concern that resistant bacteria could be transmitted between the species (Hutton 2018).

1.6 Antimicrobial resistance in dogs

A study that investigated dogs visiting a UK veterinarian practice found that many of the dogs sampled had AMR *E. coli* (44.8% of 260 dogs; Wedley *et al.* 2017). The aim of that particular study was to estimate the prevalence and investigate the molecular characteristics of ESBL and plasmid-encoded AmpC-producing *E. coli* in the UK vet-visiting canine population (Wedley *et al.* 2017). The authors provided owners with questionnaires to enable identification of potential factors associated with AMR carriage and used faecal samples obtained from the dogs to identify resistant *E. coli*. They found that recent use of antimicrobials and dogs being fed raw poultry were risk factors for AMR (Wedley *et al.* 2017).

Research has also been conducted on *E. coli* isolated from clinical samples from dogs to determine AMR. Normand and colleagues explored data from 1989-1997 and found a significant increase in individual resistance of *E. coli* carried by dogs to amoxicillin-clavulanate and streptomycin (2000). These studies provide evidence for understanding resistance, however it is also critical to examine healthy dog populations to fully understand the carriage of AMR in dogs. Research into the prevalence of faecal carriage of ESBL/AmpC-producing *E. coli* in healthy companion animals is limited (Hordijk *et al.* 2013), although there are at least two studies investigating faecal samples from healthy dogs to determine the carriage of AMR *E. coli*. Studies have investigated faecal samples from healthy dogs to determine the carriage of *E. coli* that have AMR. A study of 78 dogs in Portugal looked at possible risk factors associated with AMR and found that previous quinolone treatment and coprophagic habits were risk factors associated with the increased carriage of AMR bacteria (Leite-Martins *et al.* 2014). Another study showed that dogs are carriers of AMR *E. coli*; 183 healthy dogs in Cheshire were examined and 29% carried resistant isolates (Wedley *et al.* 2011). These studies indicate that adult dogs are carriers of resistant *E. coli.*

Dogs that are given antibiotic treatment have also been shown to carry an increased amount of resistant bacteria. The effect of oral amoxicillin treatment on seven healthy adult dogs was evaluated by looking at the faecal microbiota of the dogs (Grønvold *et al.* 2010). The prevalence of bacterial resistance and changes to the bacterial population were examined. After four to seven days of exposure to amoxicillin the faecal *E. coli* expressed resistance to multiple antibiotics compared to before the exposure to amoxicillin (Grønvold *et al.* 2010). This indicates the impact antibiotics can have on AMR in dogs and is more evidence that recent antibiotic treatment is a risk factor for the carriage of AMR *E. coli* by dogs. Overall, AMR bacteria have been detected in both healthy and sick adult dogs, associations have been found between increased AMR and exposure to antimicrobials and veterinary healthcare as well as with coprophagia and dogs being fed raw poultry (Wedley *et al.* 2017; Grønvold *et al.* 2010; Leite-Martins *et al.* 2014).

1.7 Aims and Objectives

In this study, different risk factors were investigated to explore associations between various lifestyle factors and the detection of resistant *E. coli* in puppy faecal samples. It was hypothesised that there are certain factors in the management and lives of puppies and dogs that increase or decrease the risk of carriage of AMR *E. coli.*

It was postulated that a dog's diet would be a potential risk factor and that the feeding of raw meat would increase the risk of resistant *E. coli* in the gut of the puppy or dog, as has been shown previously. Baede and colleagues suggested a possible association between AMR in faecal *E. coli* of dogs that were fed raw meat (2015) and Schmidt and colleagues isolated faecal *E. coli* from healthy Labradors in the UK and also found raw feeding dogs to be a risk factor and a potential source of AMR transmission (Schmidt *et al.* 2015).

Further hypotheses investigated in this study were that the environment in which the dog is walked would be associated with carriage of resistant *E. coli.* The impacts of puppies and dog walking in different environments such as countryside, town, farmland, beaches and near cattle were explored to see whether walking in these environments increased the risk of the dog carrying resistant *E. coli*.

Another hypothesis was that the puppy swimming and playing in water would increase the risk of carriage of AMR *E. coli*. Playing in saltwater, lake water, river water and pond water was investigated to assess whether there was a correlation between the dog swimming in these water sources and having resistant *E. coli.*

It was also postulated that rolling in cow pats or fox faeces would increase the risk of the dog carrying AMR *E. coli* and that dogs displaying autocoprophagic behaviours would be at a greater risk of having resistant bacteria.

This research also assessed whether the age of the dog had an impact on the carriage of resistant *E. coli* and compared resistance in 12-week-old puppies, 16-

week-old puppies and adult dogs. It was hypothesised that as the dog ages, the risk of carrying resistant *E. coli* would increase as the dog is exposed to more resistance in the environment. It was also possible to compare 16-week-old puppies recruited from different areas in the UK to assess any difference in resistance. It was postulated that any differences in recruitment locations would not have an impact on the carriage of AMR *E. coli* in the puppies.

Chapter 2 - Materials and Methods

2.1 Recruitment of the cohorts

Puppy and adult dog owners were recruited to take part in this study, with puppy owners recruited in two ways: (1) 236 were already recruited to the Generation Pup project, a longitudinal study looking at the health, welfare and behaviour of dogs across the UK and (2) 80 recruited via word-of-mouth advertisement to clients bringing puppies in for routine checks to veterinary practices in Somerset, North Somerset, Bath and Bristol. As part of Generation Pup, owners completed surveys relating to their puppies at 16 weeks of age. Data provided in answer to questions set out in the Appendix were extracted from the wider Generation Pup survey data. Puppy owners also supplied a single faecal sample at 16 weeks of age. Local puppies were recruited via puppy socialisation classes, social media and local media advertisement. Here, owners answered survey questions as set out in the Appendix in relation to puppies aged ≤12 weeks and again at 16 weeks. Local owners provided two faecal samples for each puppy: one at ≤12 weeks and one at 16 weeks of age. All puppy owners were recruited between August 2017 and March 2018 and all owners gave consent. Ethical approval for this study was granted by the University of Bristol Health Sciences Student Research Ethics Committee (56783). Health status of the puppies and prior veterinary treatment was not recorded. However, puppies that had been previously hospitalised were excluded.

Adult dogs were also recruited in two ways: (1) 18 adult dogs were recruited from veterinary practices and by word of mouth in Somerset, North Somerset, Bath and Bristol between October 2017 and January 2018. These adult dog owners were asked to complete a questionnaire (Appendix) and asked to provide a faecal sample from their dog. (2) 16 adult dogs were recruited from the River Thames area of Bullcroft Park, Wallingford, Oxfordshire in November 2017. These adult dog owners were asked to provide a faecal sample collected from their dog.

2.2 Faecal samples and processing

On recruitment, puppy and dog owners were supplied with a sample collection pack comprised of a specimen bottle, gloves, biohazard bag and a free post envelope. For those owners asked to provide a second sample (local puppy owners), another sample collection pack was sent to them by post. Owners of these locally recruited puppies were asked to provide fresh faecal samples as follows: the first sample before their puppy was walked in public areas and the second sample when the puppy was over 16-weeks-old and able to walk in public areas. The locally recruited and Oxfordshire adult dogs were asked to provide one faecal sample from their dog. Faecal samples were then sent by post to the University of Bristol's Veterinary School alongside the consent form and questionnaire. To process each faecal sample, approximately 0.1-0.5 g of the faecal samples was taken and weighed. 10 ml per g of phosphate buffered saline (PBS) was added to the sample and the mixture vortexed. Next, 0.5 ml of the faecal/PBS homogenate was added to 0.5 ml of 50% v/v glycerol, and samples were archived at -70°C. All faecal samples were treated in the same way.

2.3 Testing for antimicrobial resistance

To test for AMR in the *E. coli* of the faecal samples, each faecal homogenate was plated on six different Tryptone Bile X-Glucuronic Agar (TBX) agar plates. These were TBX containing no antibiotics or TBX containing either ciprofloxacin, cephalexin, amoxicillin, tetracycline or streptomycin. Onto each of these six plates, 20 μl of the faecal homogenate was spread. The breakpoints were: ciprofloxacin 0.5mg/L, tetracycline 16mg/L, amoxicillin 8mg/L, cephalexin 16mg/L, streptomycin 64mg/L (European Committee on Antimicrobial Susceptibility Testing 2018). It was necessary to use 10-fold serial dilution on some of the faecal homogenates with PBS to achieve countable numbers of colonies. The plates were incubated at 37°C overnight. The number of green/blue

E. coli colonies on each plate were counted and recorded in a database.

2.4 Levels of detection

Some puppy and adult dog faecal samples were excluded from this study as no *E. coli* were found on the plates containing no antibiotics and therefore it could not be determined whether there was resistant *E. coli* or not. A limit of detection was chosen whereby only samples where 20 or more colonies grew on agar (with no antibiotics) were retained in the analysis thereby ensuring an appropriate level of detection. Samples with a count of less than 20 cfu (colony-forming-units) on agar with no antibiotics (when 1 μg of faeces was plated) were re-plated using a larger inoculum of 5 μg (100 μl of the faecal homogenate) to test for resistance at a higher volume. If the *E. coli* count was less than 20 cfu with this larger inoculum, the samples were excluded from this study.

2.5 Risk factor analysis

A risk factor analysis was carried out on the data from 12-week-old puppies (locally recruited), 16-week-old puppies (locally recruited and Generation Pup) and adult dogs (locally recruited). The risk factor analysis was done with advice from Ashley Hammond who coded and provided the original models. The faecal samples were coded as being positive or negative for *E. coli* resistant to any antibiotic as well as positive or negative to each of the five antibiotics: ciprofloxacin, cephalexin, amoxicillin, tetracycline and streptomycin. Questionnaire data from the locally recruited dog and puppy owners along with relevant data extracted from the wider Generation Pup surveys was used in the risk factor analysis.

Univariable and multivariable logistic regression models were used to evaluate associations between resistance and risk factors identified from the survey data (Stata/IC 15.1, StataCorp LLC, College Station, TX, USA). A backward stepwise method was used. In this method the full set of possible factors were analysed,

with the least significant factors removed one-at-a-time until all remaining factors had *p*-values of 0.05 or less. Multivariable models were carried out on all of the screened 16-week-old puppy samples (n=223), however autocoprophagia, rolling in cow pats and rolling in fox faeces were excluded as these were only present in the Generation Pup data. Therefore, multivariable models were also built for the Generation Pup data alone (n=182) which could include autocoprophagia, rolling in cow pats and rolling in fox faeces as predictors.

Risk factor associations were considered statistically significant if $p<0.05$. For the risk factor analysis, it was necessary to categorise questionnaire answers as 'Yes' or 'No'; questionnaire answers of 'sometimes, often, almost always, and frequently' were all categorised as 'Yes'. Some categories were combined as part of the analysis.

The risk factors investigated were: feeding the puppy uncooked/raw food, walking the puppy in town/city, walking the puppy on farmland, walking the puppy on beaches, walking the puppy in the countryside, walking the puppy around cattle, whether the puppy had ever swum/paddled/played in salt water, whether the puppy had ever swum/paddled/played in lake water, whether the puppy had ever swum/paddled/played in river water, whether the puppy had ever swum/paddled/played in pond water, whether the puppy had ever rolled in cow pats, whether the puppy had ever rolled in fox faeces and if the puppy had displayed autocoprophagic behaviour in the past seven days.

2.6 Statistical Tests

Other statistical tests were also carried out. Pearson's chi-squared test was used to calculate the *p*-values for the baseline data from the locally recruited adult dogs comparing whether AMR was found in *E. coli* obtained from the faecal samples depending on whether the questionnaire response was 'Yes' or 'No' using Stata (Table 3.2). A *p*-value below 0.05 was considered significant. Fisher's Exact Test was used to compare whether or not resistance to each of the antimicrobials was found in the *E. coli* obtained from locally recruited 12-week-

old puppies and 16-week-old puppies and to compare whether resistance to each of the antimicrobials was or was not found in the *E. coli* in puppies recruited locally and puppies recruited through Generation Pup (Table 3.4 and Table 3.5). A *p-value* was considered significant if below 0.05.

2.7 Molecular Techniques – PCR, Whole Genome Sequencing

The 12-week-old puppy, 16-week-old puppy and adult dog samples that grew *E. coli* with resistance to cephalexin and ciprofloxacin underwent molecular procedures to provide polymerase chain reaction (PCR) and whole genome sequence data (Figure 2.1; Figure 2.2). These techniques were carried out by Jacqueline Findlay and Oliver Mounsey.

Polymerase chain reaction (PCR) is rapid technique used in molecular biology that allows the precise detection and production of large amounts of DNA and is extensively used by both researchers and clinicians to diagnose diseases, clone and sequence genes (Garibyan & Avashia 2013). The requirements for a PCR are template DNA, primers, nucleotides and DNA polymerase. Taq DNA polymerase is an enzyme isolated from *Thermus aquaticus* that joins individual nucleotides together to form the PCR product (Saiki *et al.* 1988; Garibyan & Avashia 2013).

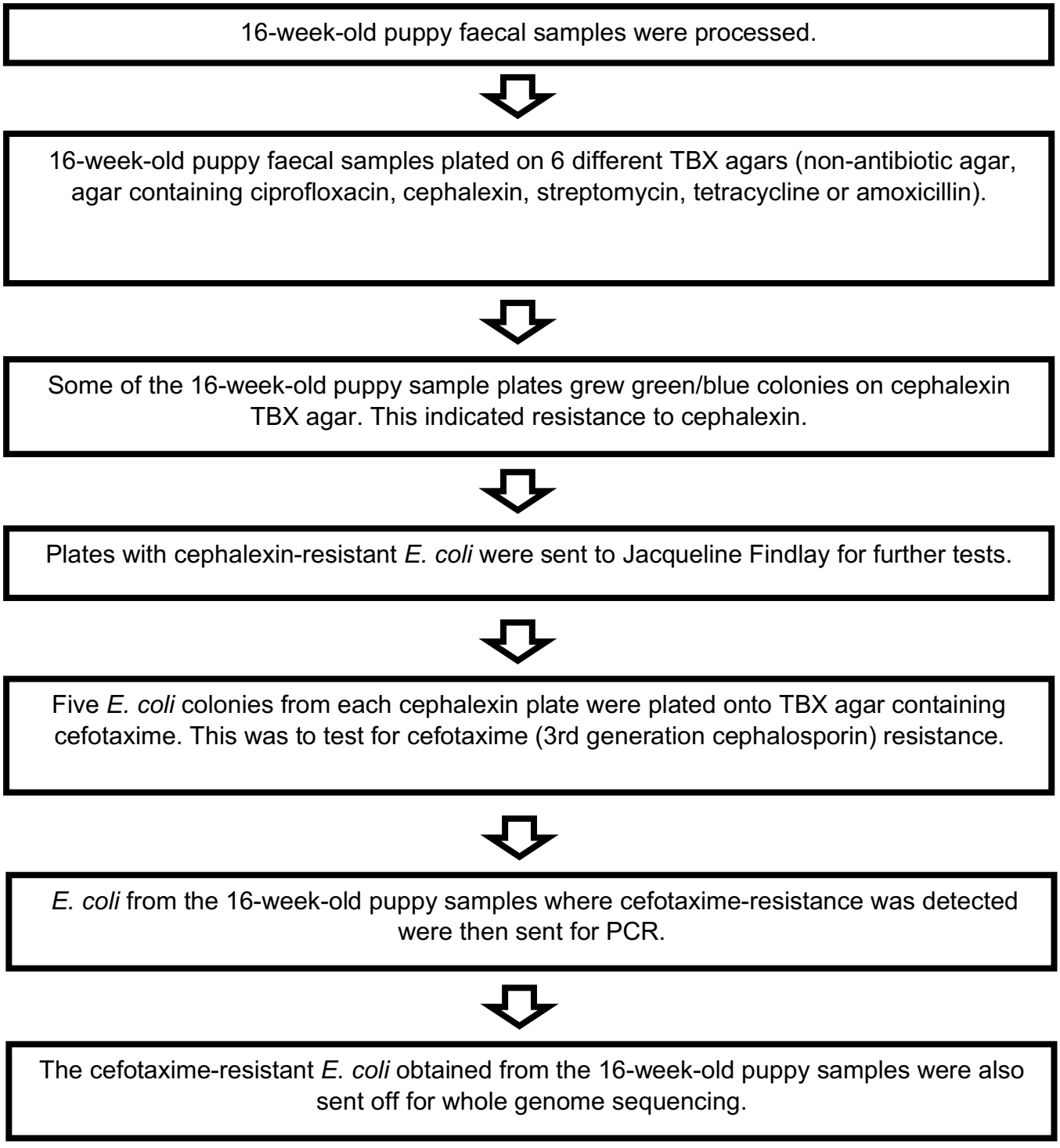


Figure 2.1. Flow diagram showing the sequence of events of processing the faecal samples, screening for cephalexin resistance, re-screening for cefotaxime resistance and further molecular testing (PCR and whole genome sequencing).

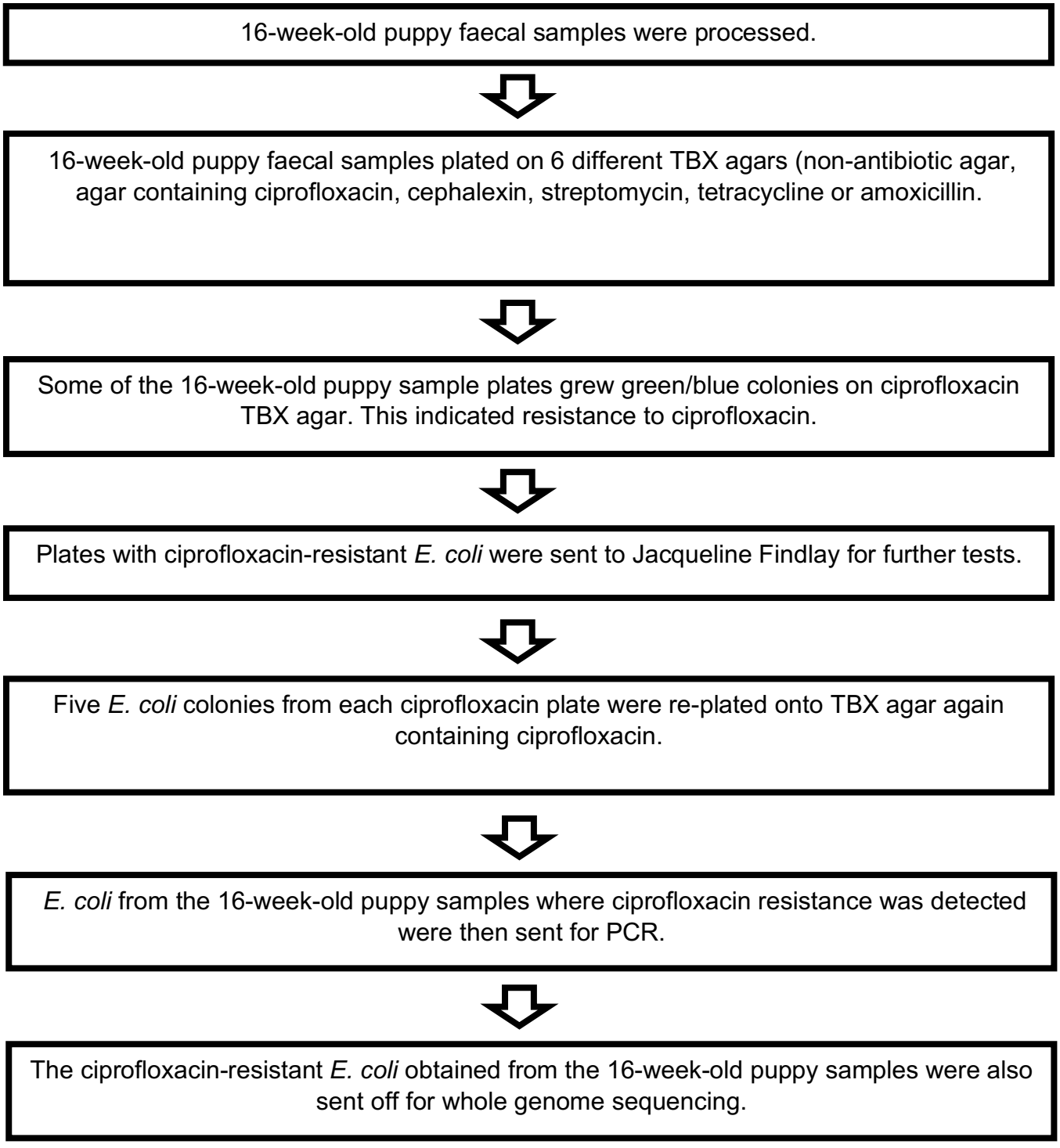


Figure 2.2. Flow diagram showing the sequence of events of processing the faecal samples, screening for ciprofloxacin resistance and further molecular testing (PCR and whole genome sequencing).

Five *E. coli* colonies from the cephalexin were re-plated by Jacqueline Findlay onto TBX agar containing cefotaxime (third generation cephalosporin) in order to select for β-lactamases, ESBLs and for AmpC production and were incubated overnight at 37°C.

Molecular tests were carried out on the cefotaxime and ciprofloxacin resistant *E. coli.* BL, CTX-M and Random Amplification of Polymorphic DNA (RAPD) PCRs were carried out. The CTX-M as well as other BL multiplexes (TEM, SHV, CMY, DHA, OXA-1) and floR primers were performed on the cefotaxime-resistant colonies. First lysates were prepared by boiling 1-2 colonies in 100 μl of water for 5 minutes at 95°C in the thermal cycler. The plates were then centrifuged at 3500 rpm for 5 minutes and 1 μl of lysate per PCR rxm were used. The conditions used for the CTX-M and RAPD PCR are shown for CTX-M multiplex (Table 2.1; Table 2.3); an annealing temperature of 62°C was required whereas for BL an annealing temperature of 56°C was required (Table 2.2).

Table 2.1. Primers for the CTX-M multiplex PCR. The primer name, sequence and product size are shown. 62°C annealing was required.

Primer	**Sequence (5'-3')**	**Product size (bp)**
Group1_F	AAAAATCACTGCGCCAGTTC	415
Group1_R	AGCTTATTCATCGCCACGTT	
Group2_F	CGACGCTACCCCTGCTATT	552
Group2_R	CCAGCGTCAGATTTTTCAGG	
Group9_F	CAAAGAGAGTGCAACGGATG	205
Group9_R	ATTGGAAAGCGTTCATCACC	
Group8_F	TCGCGTTAAGCGGATGATGC	666
Group25_F	GCACGATGACATTCGGG	
Group8/25_R	AACCCACGATGTGGGTAGC	327

Table 2.2. Primers for the BL multiplex PCR. The primer name, sequence and product size are shown. 56°C annealing was required.

Primer	Sequence (5'-3')	Product size (bp)
CMY_G1_F	CGATCCGGTCACGAAATACT	556
CMY_G1_R	CCAGCCTAATCCCTGGTACA	
DHA_F	GTGAAATCCGCCTCAAAAGA	341
DHA_R	ACAATCGCCACCTGTTTTTC	
TEM_F	CCGAAGAACGTTTTCCAATG	249
TEM_R	GTCCTCCGATCGTTGTCAGAA	
SHV_F	CTTTCCCATGATGAGCACCT	127
SHV_R	GCGAGTAGTCCACCAGATCC	
OXA_F	TTATCTACAGCAGCGCCAGT	451
OXA_R	AAGCTACTTTCGAGCCATGC	
floR_F	GCATTGATCGGCGAGTTCTT	620
floR_R	TTTAAAAGTGCCACCGCCAA	

The PCR set up required 10 µl of MyTaq mix, 0.5/1 µl of Primer mix, 8 µl of water and 1 µl of DNA. The cycling conditions were 98°C for initial denaturation with a duration of 5 minutes followed by 30 seconds of denaturation at 98°C and then annealing at 62°C or at 56°C for 35 cycles of 30 seconds for CTX-M or 30 cycles for 30 seconds for BL. An extension period of 30 seconds at 72°C was followed by a final extension for 5 minutes at 72°C. The hold conditions were kept at 10°C. Controls were also included in each run.

A RAPD PCR was also carried out on isolates, and a primer was used (Table 2.3). This PCR set-up used the same volumes as above. The cycling conditions were 5-minute initial denaturation at 98°C then 30 seconds denaturation at 98°C. This was followed by 45 cycles of 30 seconds at 36°C and then a 30 second period of extension at 72°C and a final extension for 5 minutes at 72°C. The hold temperature was 10°C.

Table 2.3. The primer for the RAPD PCR. The primer name and sequence are shown.

Primer	Sequence (5'-3')
1283	GCGATCCCCA

The cefotaxime-resistant *E. coli* colonies from the puppies and dogs were sent off for whole genome sequencing to provide further information about AMR. Multilocus sequence typing (MLST) was carried out on the *E. coli* to provide information about the Sequence Type (ST) of the *E. coli.* The whole genome sequence provides data on genome size, ST, MLST genes found as well as the resistance genes detected. The virulence genes and plasmids are also identified. STs can be identified using the Achtman Scheme (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) to see in which species they commonly occur. The whole genome sequencing data is vital in fully understanding the origins of *E. coli* found in the dogs and possible routes of transmission of the resistance. The PCR and whole genome sequencing results were returned to me and I analysed the results. The ST could be searched on a database called EnteroBase to look for other places the ST had been found previously. It was possible to identify specific resistance genes from the sequencing results.

Chapter 3 – Young puppy risk factor analysis

3.1 Introduction

AMR is a rapidly worsening global health threat that impacts the heath of animals and humans (Tacconelli *et al.* 2017). Because many serious bacterial infections are opportunistic, the carriage of AMR bacteria in normal flora of dogs is a potential source of infections that are difficult to treat in dogs as well as in the humans who interact with them (Wedley *et al.* 2011). *E. coli* is a bacterium commonly found in the intestines of dogs (Carattoli *et al.* 2005) and previous studies have highlighted the high prevalence of AMR *E. coli* found in faecal samples taken from dogs (Hordijk *et al.* 2013). *E. coli* is a primary cause of opportunistic community- and healthcare-associated infections in humans in the UK (Abernethy *et al.* 2017). Dogs live in close proximity to humans and potentially may pass on AMR *E. coli* to humans and vice versa (Costa *et al.* 2007; Guardabassi *et al.* 2004; Sidjabat *et al.* 2006). Studies examining transmission of *E. coli* within a household concluded that some *E. coli* are transmitted between household members, including dogs (Damborg *et al.* 2009; Johnson *et al.* 2008; Grönthal *et al.* 2018). It is also possible that dogs carry and spread AMR bacteria to other animal species. For instance, if dogs are found to contribute to the transmission of AMR to livestock, this could pose a problem for the food industry and global food security.

An increased understanding of the risk factors that lead to colonisation of dogs with AMR *E. coli* may help suggest possible interventions to reduce AMR. The use of antimicrobials is already a known risk factor for the acquisition of AMR in dogs (Wedley *et al.* 2011; Schmidt *et al.* 2015), however, this may not be the only driver; management practices that influence ingestion of AMR bacteria may also play a key role, particularly in early life. Accordingly, the aim of this study was to evaluate the occurrence of AMR *E. coli* in the faeces of puppies (at 16 weeks of age) and to assess potential associations between risk factors from the puppy's lifestyle that may influence the abundance of AMR *E. coli.* Of particular interest

was the influence of environmental interaction on AMR carriage.

3.2 Results and Discussion

Table 3.1. Number of faecal samples and completed surveys for locally recruited and Generation Pup (nationally recruited) puppies at ≤12 weeks and 16 weeks of age. Samples that had a limit of detection issue where the *E. coli* count was less than 20 cfu were excluded as were puppy samples that did not include a completed questionnaire.

	Locally recruited samples	**Generation Pup samples**
12-week-old puppies recruited	80	0
Screened	64	-
Excluded	16	-
16-week-old puppies recruited	59	236
Screened	41	182
Excluded	18	54

Table 3.1 shows the number of 12-week-old and 16-week-old puppies that were recruited for this study through Generation Pup and those that were locally recruited. The number of these samples that were screened and included in the results are also shown (Table 3.1). In total, 287 puppies were screened as they had completed surveys and faecal samples to test for antimicrobial resistant *E. coli* (Table 3.1). There was a surprisingly low amount of *E. coli* detected in the 16-week-old puppy faecal samples and a high number of puppy faecal samples that did not grow *E. coli* colonies on the agar plates containing no antibiotics (14 of the 16-week-old puppy samples were excluded due to incomplete questionnaires and 58 were excluded due to limit of detection; Table 3.1).

A total of 223 16-week-old puppies were screened for AMR (Generation Pup and locally recruited). The baseline questionnaire data was used to test the association of specific responses with the presence of resistance (i.e. to one or more of: amoxicillin, cephalexin, ciprofloxacin, streptomycin or tetracycline) in *E. coli* from the faecal sample using the Pearson Chi-squared test (Stata/IC 15.1, StataCorp LLC, College Station, TX, USA). A puppy being fed raw food was the only risk factor with a high level of significance (Pearson chi-squared 14.41; *p*-value 0.001; Table 3.2). Some possible risk factors (autocoprophagia, rolling in cow pats and rolling in fox faeces) which looked promising based on initial analyses could only be obtained from the Generation Pup data (not the locally recruited dogs) because that survey was more extensive. As part of the wider Generation Pup survey, the puppy owners were asked whether their 16-week-old puppy had displayed autocoprophagic behaviour in the past seven days and this data was included in the analysis. These data are presented separately (Table 3.3). Again, in the Generation Pup data only, puppies being fed raw food was a significant risk factor (Pearson chi-squared 9.82; *p*-value 0.002) and puppies displaying autocoprophagic behaviours in the past seven days was protective (Pearson chi-squared 6.56; *p*-value 0.01; Table 3.3)

Table 3.2. Baseline data for all 16-week-old puppies (Generation Pup and locally recruited; n=223) and associations with risk factors for AMR. *P*-values were calculated using the Pearson Chi-squared test. The bold figures show a *p*-value < 0.05.

Risk factor from questionnaire	**Response to question**	**Response to question total (n=223)**	**Also resistant to any antibiotic (n=106)**	***p*-value**
Fed raw food	Yes	43	32/43	**<0.001**
	No	180	76/180	
Walked in town	Yes	181	84/181	0.21
	No	42	24/42	
	Yes	142	69/142	0.95

Risk factor from questionnaire	**Response to question**	**Response to question total (n=223)**	**Also resistant to any antibiotic (n=106)**	***p*-value**
Walked on farmland	No	81	39/81	
Walked on beaches	Yes	103	52/103	0.57
	No	120	56/120	
Walked in the countryside	Yes	191	95/191	0.34
	No	32	13/32	
Walking near cattle	Yes	84	37/70	0.31
	No	139	71/139	
Swum/ paddled/ played in salt water	Yes	62	32/62	0.56
	No	161	76/161	
Swum/ paddled/ played in lake water	Yes	29	17/29	0.24
	No	194	91/194	
Swum/ paddled/ played in river water	Yes	66	33/66	0.76
	No	157	75/157	
Swum/ paddled/ played in pond water	Yes	65	38/65	0.06
	No	158	70/158	

Table 3.3. Baseline data for 16-week-old puppies recruited through Generation Pup (n=182) and associations with risk factors for AMR. *P*-values were calculated using the Pearson Chi-squared test. The bold figures show a *p*-value < 0.05.

Risk factor from questionnaire	**Response to question**	**Response to question total (n=182)**	**Also resistant to any antibiotic (n=94)**	***p*-value**
Fed raw food	Yes	41	30/41	**0.002**
	No	141	64/141	
Walked in town	Yes	141	70/141	0.32
	No	41	24/41	
Walked on farmland	Yes	119	61/119	0.89
	No	63	33/63	
Walked on beaches	Yes	80	42/80	0.84
	No	102	52/102	
Walked in the countryside	Yes	165	86/165	0.69
	No	17	8/17	
Walking near cattle	Yes	68	31/68	0.21
	No	114	63/114	
Swum/ paddled/ played in salt water	Yes	50	29/50	0.29
	No	132	65/132	
Swum/ paddled/ played in lake water	Yes	26	15/26	0.51
	No	156	79/156	
Swum/ paddled/ played in river water	Yes	56	29/56	0.98
	No	126	65/126	
Swum/ paddled/ played in pond water	Yes	65	38/65	0.11
	No	124	59/124	
Rolled in cow pats	Yes	6	5/6	0.11

Risk factor from questionnaire	**Response to question**	**Response to question total (n=182)**	**Also resistant to any antibiotic (n=94)**	***p*-value**
	No	176	89/176	
Rolled in fox faeces	Yes	9	4/9	0.66
	No	173	90/173	
Displayed autocoprophagic behaviour in past seven days	Yes	15	3/15	**0.01**
	No	167	91/167	

Univariable and multivariable logistic regression analyses were carried out on the data from the 16-week-old puppies in order to investigate more deeply the potential risk factors identified as being significant or tending to significance in the preliminary analysis (Table 3.4). A strong association was demonstrated between feeding a 16-week-old puppy raw food and the carriage of *E. coli* with resistance to any of the antibiotics as well as individually with resistance to all five of the antibiotics tested (Table 3.4). An example of this association is that puppies that were raw food had between 5.01 to 30.78 greater odds of having ciprofloxacin-resistant *E. coli* than puppies that were not raw fed (Multivariable: 12.42 (5.01 to 30.78) $<$0.001; Table 3.4).

This is evidence that puppies that are raw fed have an increased risk of carrying AMR *E. coli*. This link has also been previously reported. A study based of 445 dogs found that feeding raw poultry significantly increased the risk of carrying fluoroquinolone-resistant *E. coli* in the UK adult dog population (Wedley *et al.* 2017). A study of Labradors also found an association between dogs who ate raw food and amoxicillin resistance (Schmidt *et al.* 2015). A study on *E. coli* from faecal samples taken from broilers at a slaughterhouse also detected ciprofloxacin resistance which may have been due to the usage of fluoroquinolones in the production of broilers (Costa *et al.* 2009) and this could be a potential source of resistance in dogs having been fed raw food. Raw chicken imported to the UK has also been identified as a source of

fluoroquinolone-resistant *E. coli* (Warren *et al.* 2007) and AMR *E. coli* have been found in uncooked chicken carcasses (Randall *et al.* 2011; Machado *et al.* 2008). The risk of puppies acquiring AMR bacteria from raw food could be mitigated by cooking meat in order to reduce contamination and colonisation of the gut with such bacteria. It may also be possible that the raw food diet creates a gut environment which is selective for AMR *E. coli.*

It is also possible that people can acquire AMR bacteria from puppies and some have hypothesised that companion animals may act as reservoirs for AMR bacteria and may transmit these to humans, hence a One Health approach needs to be taken (Ewers *et al.* 2012; Gandolfi-Decristophoris *et al.* 2013; Timofte *et al.* 2016). Due to the close proximity of dogs to humans and other domestic pets, it is feasible that AMR bacteria could be easily transmitted by owners coming into contact with faecal matter from their dog. Owners that raw feed their dog may be at greater risk through handling raw food contaminated with AMR bacteria, but also because AMR bacteria are being shed in the faeces of their raw-fed dog. Several studies have promoted the theory that the spread of (clinically relevant) multi-resistant ESBL-producing *E. coli* has a zoonotic potential (e.g. between dogs, poultry and humans) and that a One Health approach is needed to prevent this (Overdevest *et al.* 2011; Mora *et al.* 2010; Schaufler *et al.* 2015).

Swimming in lake water was shown to be a potential risk factor for ciprofloxacin resistance (Univariable: 3.72 (1.44 to 9.61) 0.007; Table 3.4). Swimming in pond water was a potential risk factor for tetracycline resistance (Univariable: 1.80 (1.00 to 3.25) 0.05; Table 3.4) and amoxicillin resistance (Multivariable: 1.91 (1.05 to 3.48) 0.04; Table 3.4). Swimming in pond water also showed a trend for resistance to any of the antibiotics (Multivariable: 1.66 (0.91 to 3.04) 0.10). It can therefore be hypothesised that swimming in water may be a risk factor for 16-week-old puppies carrying resistant *E. coli* and further investigation into this potential risk factor should be carried out. In Chapter 4 a comparison is made between adult dogs recruited from an area close to the River Thames (Oxfordshire) to another cohort of dogs recruited around Bristol in order to evaluate whether playing in water has an impact on AMR.

Some risk factors were only investigated in the 16-week-old puppies recruited via Generation Pup (n=182; Table 3.5) as data on these risk factors was not available for locally recruited puppies. Puppies that rolled in cowpats (six were identified in the Generation Pup cohort) were shown to have an increased risk of carrying cephalexin-resistant *E. coli* (Multivariable: 5.52 (1.06 to 28.79) 0.04; Table 3.5) or streptomycin-resistant *E. coli* (Multivariable: 11.42 (1.51 to 86.17) 0.02; Table 3.5). A possible explanation for this is that there is a fitness advantage for streptomycin-sensitive versus streptomycin-resistant strains to be carried in dogs in the absence of selection (Frost *et al.* 2018). Generally interacting with cattle as part of the wider environment may provide for a more complex microbiological flora, where competition can occur and resistance may be reduced. Rolling in cowpats may predispose dogs towards a more restricted flora where competition is less and therefore resistance can thrive. More work is needed to investigate this potential risk factor, and research is currently underway at the University of Bristol determining the levels of AMR *E. coli* in cattle faeces to find patterns and possible risk factors which may contribute to evaluating this risk factor.

Puppies that displayed autocoprophagic behaviour in the seven days prior to sampling had a reduced risk of carrying *E. coli* with resistance to any one of the antibiotics (Generation Pup recruited; Multivariable: 0.22 (0.06 to 0.83) 0.03; Table 3.5) as well as reduced risk of specifically carrying tetracycline- and amoxicillin-resistant *E. coli* (Table 3.5). The observation that autocoprophagia reduces the risk of puppies and dogs carrying AMR *E. coli* has not been identified previously. At least one other study found that dogs showing coprophagic behaviour (eating either their own faces or faeces from other animals such as livestock) were at increased risk of carrying AMR bacteria (Leite-Martins *et al.* 2014). However, this study only considered adult dogs not puppies and looked at both autocoprophagy and allocoprophagy. These differences in populations could be an explanation for the difference in the results. It could be postulated that autocoprophagy has a different impact on young puppies compared to adult dogs due to differences in gut flora however, further research would need to be conducted.

The risk factor analysis generated possible hypotheses which could explain

resistance in the *E. coli* carried by the 16-week-old puppies. Some potential risk factors were not significant (>0.05) in the multivariable analyses, however, they may reveal trends for further investigation. Walking around town showed a trend for ciprofloxacin resistance (Multivariable: 4.66 (0.96 to 22.64) 0.06) in the whole dataset but it was significant in the Generation Pup data (Multivariable: 4.83 (1.00 to 23.39) 0.05; Table 3.5). This could suggest that there is less walking around town in the local cohort compared to the Generation Pup cohort. In the Generation Pup data, playing in salt water also showed a trend for tetracycline resistance (Multivariable: 1.86 (0.93 to 3.73) 0.08). All of these trends require further investigation to establish whether they are risk factors for AMR.

Table 3.4. Univariable and multivariable logistic regression analyses using questionnaire data and AMR *E. coli* data for 16-week-old puppies (recruited via Generation Pup and locally recruited), excluding samples with a limit of detection issue where the *E. coli* count was less than 20 cfu or missing or incomplete questionnaires (n=223). Presentation: Odds ratio (95% confidence interval) *p*-value. Only significant risk factors are shown the full results are present in the appendix. The bold figures indicate a significant *p*-value p<0.05.

Risk Factor	**Univariable (n=223)**	**Multivariable for all samples (n=223)**	**Multivariable for Generation Pup Samples (n=182)**
Resistance to any antibiotic (n=108)			
Fed raw food	**3.98 (1.89 to 8.40) <0.001**	**3.98 (1.89 to 8.40) <0.001**	**3.20 (1.47 to 6.96) 0.003**
Resistance to ciprofloxacin (n=26)			
Fed raw food	**12.42 (5.01 to 30.78) <0.001**	**12.42 (5.01 to 30.78) <0.001**	**11.90 (4.47 to 31.64) <0.001**
Walked around town	3.06 (0.69 to 13.48) 0.14	4.66 (0.96 to 22.64) 0.06	**4.83 (1.00 to 23.39) 0.05**
Swum/paddled/ played in in lake water	**3.72 (1.44 to 9.61) 0.007**	1.28 (0.39 to 4.20) 0.69	0.98 (0.27 to 3.52) 0.97
Walked on	**2.44 (1.04 to**	1.70 (0.66 to 4.42)	1.79 (0.65 to

Risk Factor	**Univariable (n=223)**	**Multivariable for all samples (n=223)**	**Multivariable for Generation Pup Samples (n=182)**
beaches	**5.74) 0.04**	0.27	4.92) 0.26
Resistance to tetracycline (n=81)			
Fed raw food	**4.47 (2.21 to 9.05) <0.001**	**4.47 (2.21 to 9.05) <0.001**	**3.52 (1.67 to 7.40) 0.001**
Swum/ paddled/ played in pond water	**1.80 (1.00 to 3.25) 0.05**	1.68 (0.91 to 3.12) 0.10	1.657 (0.75 to 3.27) 0.23
Resistance to amoxicillin (n=93)			
Fed raw food	**3.30 (1.64 to 6.63) 0.001**	**3.18 (1.57 to 6.42) 0.001**	**2.55 (1.23 to 5.30) 0.01**
Swum/ paddled/ played in pond water	**2.01 (1.12 to 3.61) 0.02**	**1.91 (1.05 to 3.48) 0.04**	1.80 (0.93 to 3.47) 0.08
Resistance to cephalexin (n=34)			
Resistance to streptomycin (n=51)			
Fed raw food	**8.23 (3.95 to 17.15) <0.001**	**8.23 (3.95 to 17.15) <0.001**	**6.21 (2.81 to 13.72) <0.001**

Table 3.5. Univariable and multivariable logistic regression analyses using questionnaire data and AMR *E. coli* data for 16-week-old puppies recruited via Generation Pup, excluding samples with a limit of detection issue where the *E. coli* count was less than 20 cfu or missing or incomplete questionnaires (n=223). Only significant results are shown, the full results are present in the appendix. Presentation: Odds ratio (95% confidence interval) *p*-value. A *p*-value was considered significant if $p<0.05$.

Risk Factor	**Univariable (n=182)**	**Multivariable for Generation Pup Samples (n=182)**
Resistance to any antibiotic (n=94)		
Autocoprophagic behaviour in past seven days	0.28 (0.09 to 0.90) 0.03	0.22 (0.06 to 0.83) 0.03
Resistance to ciprofloxacin (n=24)		
Resistance to tetracycline (n=72)		
Autocoprophagic behaviour in past seven days	0.19 (0.04 to 0.88) 0.03	0.10 (0.01 to 0.80) 0.03
Resistance to amoxicillin (n=82)		
Autocoprophagic behaviour in past seven days	0.25 (0.07 to 0.91) 0.04	0.18 (0.04 to 0.82) 0.03
Resistance to cephalexin (n=30)		
Rolled in cow pats	5.56 (1.06 to 28.98) 0.04	5.52 (1.06 to 28.79) 0.04
Resistance to streptomycin (n=51)		
Rolled in cow pats	6.63 (1.17 to 37.53) 0.03	11.42 (1.51 to 86.17) 0.02

In conclusion, this research has identified factors such as raw feeding and rolling in cow pats as risks for carrying AMR *E. coli* in 16-week-old puppies. It is essential that puppy owners fully understand the risk that these practices - especially raw

feeding - pose to the health of their puppy, other animals, themselves and their contribution to the global problem of AMR. Owner education is essential, and suggestions to mitigate this risk should be encouraged (i.e. owners should be instructed to feed their puppies cooked meat or dry food instead of raw food). It may also be necessary to decrease the use of antibiotics used in food-producing animals in order to decrease the risk of AMR bacteria being transmitted to dogs as well as regulate the importation of raw meat used for feeding dogs. The strategy of the Responsible Use of Medicines in Agriculture (RUMA) Alliance is that the health and welfare of food-producing animals is important but that antimicrobials should be used responsibly. RUMA also suggest that good management practices can reduce disease which helps reduce the need for antimicrobials (RUMA 2012). Furthermore, they suggest that antimicrobials should be prescribed by a veterinary surgeon and that a full course of treatment should be completed, as well as that critically important antimicrobials should never be given preventively or as the first treatment for livestock (RUMA 2012). It is also important that a strategy is in place for responsible use of antimicrobials in small animals, the BSAVA (British small animal veterinary association) recommend the PROTECT guidelines to promote responsible antimicrobial prescribing. For example, these guidelines advise veterinarians that they should consider other options for treatment before prescribing antimicrobials, ensure the correct antimicrobial is used and ensure that treatment is carried out effectively (BSAVA 2018).

Further research could be conducted into the brands and sources of the raw food that was fed to the puppies in this study in order to assess whether all raw food products are as much of a risk for resistance carried by puppies. This could be done by surveying puppy owners that raw feed their dogs. It would also be beneficial to investigate AMR in the owners of the puppies to evaluate whether resistance is being spread between members of the household. Further research is also needed to further evaluate the hypothesis that autocoprophagic habits in the young puppies was protective against resistance to assess the reasons behind this. There were some other trends and hypotheses identified by the risk factor analysis which all need to be investigated to assess whether the factors are actual risk factors for the carriage of AMR in dogs.

Chapter 4 – Adult dogs and comparison of cohorts

4.1 Introduction

In this chapter, AMR in adult dogs was investigated and comparisons were made between adult dogs recruited from different areas in the UK. In Chapter 3 there was an indication that swimming in lake water was a risk factor for ciprofloxacin resistance (Univariable: 3.72 (1.44 to 9.61) p=0.007) and playing in pond water was a risk factor for both amoxicillin resistance (Multivariable: 1.91 (1.05 to 3.48) p=0.04) and tetracycline resistance (Univariable: 1.80 (1.00 to 3.25) p=0.05) in 16-week-old puppies. This suggested that interaction with different types of water may increase the risk of AMR in young puppies. Hence, a preliminary cohort of adult dogs was recruited to assess whether swimming in water sources was a risk factor for carrying AMR *E. coli*. Previous studies have found a correlation between humans that surf in UK coastal salt water and gut carriage of AMR bacteria, presumably as a result of accidental ingestion (Leonard *et al.* 2017). It is therefore possible that dogs that ingest pond/river/sea water may be at a greater risk of carrying AMR *E. coli*. A cohort of adult dogs that were locally recruited were compared to the adult dogs recruited from Oxfordshire to assess regional differences. Regional differences in the locally recruited 16-week-old and Generation Pup-recruited 16-week-old puppies (nationally recruited) were also compared.

Furthermore, in Chapter 3 the research focused on puppies aged 16 weeks to explore risk factors associated with AMR. Therefore, this chapter considered AMR in a cohort of recruited adult dogs to assess whether there was a difference in the carriage of AMR *E. coli* in the adult dogs compared to the young puppy cohorts. In the UK, common veterinary advice is not to walk puppies under 12 weeks of age in public places as puppies are usually not fully vaccinated. By 16 weeks of age, vaccinations are normally complete and puppies are usually walked freely in public places. Therefore, for a subgroup of puppies, comparisons in this study were also made between the levels of AMR *E. coli* in faeces from puppies ≤12-weeks of age versus 16-weeks of age to capture the influence of this initial interaction with the wider environment on AMR carriage. It is possible

that the age of a dog affects the amount of AMR *E. coli* that the dog has in its gut and very few studies have investigated whether age affects AMR carriage in dogs. Hence, in this study AMR in the *E. coli* obtained from puppies at 16-weeks-old were compared with that of adult dogs. The methods used are shown in Chapter 2.

4.2 Results and Discussion

4.2.1 Adult dogs

A total of 34 adult dogs were recruited for this study (18 locally recruited, 16 recruited from the Oxfordshire catchment area). The 18 locally recruited dog owners completed questionnaires, and these were used to determine possible risk factors for AMR *E. coli* carriage in faeces. Only 14 of these 18 dogs were screened for AMR *E. coli*; four were excluded due to a limit of detection issue where the *E. coli* count was less than 20 cfu. Of the 16 dogs recruited from the Oxfordshire area, only 11 were screened for AMR due to the same issue around limits of detection (Table 4.1).

Table 4.1 Number of faecal samples and completed samples for locally recruited and Oxfordshire adult dogs. Samples that had a limit of detection issue where the *E. coli* count was less than 20 cfu were excluded as were adult samples that did not include a completed questionnaire.

	Locally Recruited Dogs	**Oxfordshire Dogs**
Adult Dogs	18	16
Screened	14	11
Excluded	4	5

The locally recruited adult dog faecal samples were tested for AMR *E. coli* and these results were paired with the questionnaire responses. Pearson chi-squared tests were performed as initial analyses and there were no significant risk factors found when comparing responses from the questionnaire with whether or not the sample had AMR *E. coli* (Table 4.2). Univariable and multivariable logistic regressions were also carried out on the locally recruited adult dogs and no significant risk factors were found (Appendix). This part of the study may have been limited in power - a larger cohort of adult dogs may have provided evidence for potential risk factors associated with AMR.

Table 4.2 Baseline data for locally recruited adult dogs (n=14) showing potential risk factors from questionnaire responses and faecal AMR E. coli resistance. *p*-values were calculated using Pearson chi-squared tests. A *p*-value <0.05 was considered significant.

Risk factor from questionnaire	**Response to question**	**Response to question total (n=14)**	**Also resistant to any antibiotic (n=5)**	***p*-value**
Fed raw food	Yes	1	1/1	0.23
	No	13	5/13	
Walked around roads and streets	Yes	10	3/10	0.12
	No	4	3/4	
Walked in parks	Yes	6	2/6	0.53
	No	8	4/8	
Walked on beaches	Yes	10	10/4	0.42
	No	3	2/3	
	Yes	14	6/14	/

Risk factor from questionnaire	**Response to question**	**Response to question total (n=14)**	**Also resistant to any antibiotic (n=5)**	***p*-value**
Walked in the countryside without animals	No	0	0	
Walked in the countryside with other animals present	Yes	12	5/12	0.83
	No	2	1/2	
Walked in the countryside with cattle present	Yes	12	5/12	0.83
	No	2	1/2	
Swum/ paddled/ played in salt water	Yes	6	2/6	0.53
	No	8	4/8	
Swum/ paddled/ played in lake water	Yes	5	2/5	0.87
	No	9	4/9	
Swum/ paddled/ played in river water	Yes	9	5/9	0.20
	No	5	1/5	
Swum/ paddled/ played in pond water	Yes	7	4/7	0.28
	No	7	2/7	
Recently had antibiotics	Yes	2	0/2	0.19
	No	12	6/12	
Walked frequently around cattle	Yes	11	4/11	0.35
	No	3	2/3	

4.2.2 Regional comparison in adult dogs

The two differently recruited adult dog cohorts (Locally recruited and Oxfordshire recruited adult dogs) could be compared for resistance to the five different antibiotics (ciprofloxacin, tetracycline, amoxicillin, cephalexin and streptomycin) in *E. coli* found in the faecal samples (Table 4.3). A significant difference in amoxicillin resistance was shown between the cohorts - the Oxfordshire dogs were more likely to carry *E. coli* with amoxicillin resistance compared to the locally recruited dogs (p=0.02; Table 4.3).

A possible hypothesis to explain the significant difference in amoxicillin resistance could be that the Oxfordshire dogs were recruited in an area close to river water and the ingestion of water may be a risk factor for carrying *E. coli* with amoxicillin resistance. A paper examining the impact of human surfers swimming in UK coastal water found that surfers were at risk of exposure and colonisation by AMR *E. coli* (Leonard *et al.* 2017). These authors indicated that there was a possibility that the natural environment played a role in the transmission of AMR bacteria and that natural waters may act as important reservoirs of AMR bacteria (Leonard *et al.* 2017). If natural waters such as rivers, sea and lakes act as reservoirs for resistant bacteria it is possible that dogs swimming in the River Thames could be ingesting water contaminated with resistant *E. coli.* This could potentially increase the prevalence of amoxicillin-resistant *E. coli* in the gut of adult dogs that are recruited in close proximity to the River Thames compared to dogs that were recruited in the Bristol area. However, this would require further research into the impact a water environment could have on AMR and more work would need to compare the environments in the different regions.

It could be postulated that the difference in amoxicillin resistance in the Oxfordshire-recruited dogs compared to the locally recruited dogs could be because of differences in veterinary practices in those areas. The locally recruited dogs were predominately recruited through one veterinary practice and their antimicrobial prescription policies may influence the amount of amoxicillin-resistant bacteria found in the adult dog population in that region. Furthermore, the Oxfordshire dogs were recruited from a relatively small area and therefore it is possible that adult dogs went to the same veterinary practices and their

antimicrobial prescription policies may have influenced the amount of amoxicillin given to that dog population. Again, we did not collect data to test this hypothesis, but evidence has been found that AMR develops in response to treatment and that antimicrobials are frequently prescribed to small animals, including dogs (Trott *et al.* 2004; Singleton *et al.* 2017). Recent studies in the UK indicated that 25% of dogs seen at veterinary practices received at least one antimicrobial prescription (Singleton *et al.* 2017; Buckland *et al.* 2016). Research into antimicrobial prescribing in small animals also found that amoxicillin-clavulanate was the most commonly prescribed antimicrobial in small animal practices in the UK (Radford *et al.* 2011). Amoxicillin was the second most prescribed antimicrobial (20% of total prescriptions), however, these authors did find variation between different practices in the amount and types of antimicrobials being prescribed (Radford *et al.* 2011). A possible hypothesis for the results of this study, therefore, could be that the locally recruited dog veterinary practices do not prescribe amoxicillin as often as the veterinary practices in comparison to the Oxfordshire recruitment area. This might result in selection for amoxicillin-resistant bacteria in the Oxfordshire adult dog population.

The high levels of amoxicillin and amoxicillin-clavulanate prescriptions for dogs in the UK may contribute to AMR in the UK (especially amoxicillin resistance). Clavulanic acid is an inhibitor of TEM and SHV β-lactamases, which cause amoxicillin resistance (Sulton *et al.* 2005), therefore it is unlikely that these resistance genes would be detected in amoxicillin-resistant *E. coli* if the usage of amoxicillin-clavulanate contributes to amoxicillin resistance in the *E. coli* carried by dogs. Instead, amoxicillin-clavulanate use is likely to select for β-lactamases that are not inhibited by clavulanic acid, such as AmpC enzymes. This is likely to occur due to chromosomal mutations on the *ampC* promoter causing AmpC hyperproduction or due to production of a plasmid-derived CMY enzyme. These possibilities are explored in Chapter 5.

There were no significant differences in resistance to any of the other antibiotics in *E. coli* carried by the local dogs and Oxfordshire dogs (Table 4.3); the difference in AMR between the two areas was only detected with regards to amoxicillin. This could be because it was not possible to detect any differences due to the power of the study, or because the difference is exclusive to

amoxicillin.

Table 4.3 Differences in the carriage of *E. coli* resistant to five different antibiotics (ciprofloxacin, tetracycline, amoxicillin, cephalexin and streptomycin) in faecal samples from adult dogs recruited locally and from the Oxfordshire. *P*-values were calculated using Fisher's Exact Test.

	Locally recruited adult dogs	**Oxfordshire recruited dogs**	***p*-value**
Any antibiotic	6/14	8/11	0.14
Ciprofloxacin	2/14	3/11	0.62
Tetracycline	4/14	5/11	0.43
Amoxicilin	3/14	8/11	**0.02**
Cephalexin	3/14	1/11	0.60
Streptomycin	3/14	4/11	0.66

4.2.3 Regional comparison in 16-week-old puppies

Some of the puppies recruited for this study were specifically recruited in the local area (Somerset, North Somerset, Bath and Bristol) although the majority of puppies were recruited throughout the UK by Generation Pup (Chapter 3). However, all samples were obtained when the puppy was 16-weeks-old and were processed and treated in exactly the same way. It was therefore possible to compare the number of puppies carrying *E. coli* with resistance to the five antibiotics to see whether there was a difference in the amount of resistance between the two groups. W*e* compared the number of 16-week-old puppy samples that had resistant *E. coli* from the locally recruited cohort of puppies with the nationally recruited puppies (Generation Pup; Table 4.4). The results showed a significant difference in amoxicillin and tetracycline resistance in the *E. coli* obtained from locally recruited 16-week-old puppies and Generation Pup-recruited puppies. The latter had a higher proportion of puppies with amoxicillin

(*p*=0.04; Table 4.4) and tetracycline (*p*=0.05; Table 4.4) resistance. This regional difference in AMR could be due to differences in antimicrobial prescribing policy by veterinary practices in the local area compared to the national policy. For example, local veterinary practices may prescribe less amoxicillin and tetracycline compared to the national average. In order to investigate this, further research could compare the prescription data of the veterinary practices. However, this difference could be due to other regional differences in the environment and in the dog population. This regional difference in amoxicillin was also found in the adult dogs (Table 4.3).

Table 4.4 Number of puppy faecal samples carrying *E. coli* resistant to any of the five different antibiotics (ciprofloxacin, tetracycline, amoxicillin, cephalexin and streptomycin) from 16-week-old locally recruited puppies to nationally recruited (Generation Pup) puppies. *P*-values were calculated using Fishers Exact Test.

	Locally recruited puppies (16-weeks-old) with resistance	**Generation Pup nationally recruited puppies (16-weeks-old) with resistance**	***p*-value**
Ciprofloxacin	2/41	24/182	0.18
Tetracycline	9/41	72/182	**0.05**
Amoxicilin	11/41	82/182	**0.04**
Cephalexin	4/41	30/182	0.34
Streptomycin	6/41	45/182	0.22

4.2.4 Comparison of AMR *E. coli* in different ages of dogs that were locally recruited

As part of the wider study presented here, dogs were recruited at three different ages in order to investigate whether or not age affects AMR *E. coli* carriage. All puppies and dogs were recruited from the same local area. It was therefore

possible to compare resistance in the locally recruited 12-week-old and locally recruited 16-week-old puppies with resistance in locally recruited adult dogs. Samples from 12-week-old and 16-week-old puppies were compared from the same group of animals, however, adult dogs were a separate cohort.

In the UK, puppies are recommended to receive a course of core vaccinations in order to provide them with life-long protection against some infectious diseases (canine distemper virus, canine adenovirus, canine parvovirus type 2 and other variants). In the first weeks of life, puppies are protected by maternally derived antibodies but this diminishes by 8-12 weeks of age to a level that allows active immunization. It is currently recommended that puppies receive a core vaccination at 6-8 weeks of age and then every 2-4 weeks until 16 weeks of age (Day *et al.* 2016*).* Puppy owners are usually advised by veterinarians to not walk their puppy outside in public places until after the puppy has had its second vaccination (approximately 12 weeks of age). Due to this recommendation, the majority of puppies under 12 weeks have not been walked outside in public places, although at 16 weeks puppies are vaccinated and able to walk in public places. It was therefore possible and interesting to assess whether walking in public places affects the amount of resistant *E. coli* puppies carry.

The puppies at 12-weeks-old were compared with the same puppies at 16-weeks-old to see whether the puppies gained or lost AMR *E. coli* carriage. (Table 4.5). The data showed that there is a significant difference in carriage of amoxicillin-resistant *E. coli* at 12 weeks compared to 16 weeks ($p<0.001$). The same cohort of puppies at 12-weeks-old were more likely to be carrying amoxicillin resistant *E. coli* than at 16-weeks-old. A longitudinal study would need to be conducted to fully investigate this. There was no significant difference for resistance to any other antimicrobial. When comparing the resistance of the *E. coli* obtained from each puppy's faecal sample individually, there was no correlation between puppies carrying resistance to any of the five antimicrobials at 12 weeks compared to 16 weeks.

If guidelines are being followed, it might be expected that the 12-week-old puppies are exercising less (if at all) in public places than the 16-week-old puppies, so it was interesting to find that these young puppies are more likely to

carry amoxicillin-resistant *E. coli*.

This indicates that puppies have already been exposed to AMR bacteria and have acquired AMR *E. coli* very early in life, potentially from their mothers during birth or in early life. A previous investigation of spread of AMR *E. coli* amongst puppies in breeding kennels found that resistant bacteria spread between puppies in the same litter as well as amongst puppies bred and raised in close proximity (Harada *et al.* 2011). Perhaps puppies are directly receiving amoxicillin-resistant *E. coli* from their mothers which remain in their gut but diminish as the puppy ages. Future work to test this could be to take a number of different puppies from birth and test for amoxicillin resistant *E. coli* regularly to evaluate whether the move to the domestic home influences resistance.

A possible hypothesis is that puppies acquire amoxicillin-resistant bacteria at birth, however, the amount of resistant bacteria is amplified whilst in the litter, either through human contact and handling or due to changes in the gut of the puppy that select for *E. coli* with amoxicillin resistance. It could be postulated that the move from the litter to the domestic environment with different humans that handle the puppy may amplify amoxicillin resistance in the *E. coli* in puppies at 12 weeks, but that this decreases over time. One reason for this could be because the humans are transmitting amoxicillin-resistant *E. coli* to the puppies and creating a selection pressure for amoxicillin resistance in the *E. coli* carried by the puppies.

Research into AMR in European dogs has suggested that a large number of dog breeders frequently treat bitches with antimicrobials before and after they give birth with the aim of eliminating bacterial flora and reducing neonatal mortality; however, the consequence of this has been shown to be selection for resistant bacteria (Milani *et al.* 2012). The most commonly prescribed antimicrobials for this purpose were found to be amoxicillin-clavulanic acid or amoxicillin (Milani *et al.* 2012) and this could be a possible explanation for the high levels of amoxicillin resistance in young puppies. Further research would need to be conducted to test all of these hypotheses.

Further possible explanations could be that there is a correlation between the age of the dog and prescription of antimicrobials by veterinary practices; a

previous study found that antimicrobial usage decreased with an animal's age (Radford *et al.* 2011). Therefore, it is conceivable that the 12-week-old puppy population was more likely to receive antimicrobials (including amoxicillin) compared to 16-week-old puppies; this could increase amoxicillin resistance found in the *E. coli* obtained from the faecal samples.

However, it is clear that amoxicillin resistant *E. coli* colonises the puppies before 12-weeks of age and that by 16-weeks-old the number of puppies carrying amoxicillin resistance has dramatically decreased (Table 4.5). The reason for this decrease is not certain, but it may be to do with the puppies' exercising in public places and therefore presumably being exposed to a greater variability of bacteria compared to puppies at 12 weeks who are still mostly with their littermates. This could increase competition, reducing the abundance of amoxicillin-resistant bacteria in the puppy in the absence of selection (Table 4.5). The results did not show a significant difference in the number of puppies with *E. coli* showing resistance to the other antibiotics at 12 weeks compared to 16 weeks, suggesting that whatever happens to select for amoxicillin resistance does not select for resistance to the other drugs. In fact, there is a reduction of resistance in all cases, so perhaps the reality here is that whatever is happening for amoxicillin is far stronger than what is happening for other agents. It is important to note that amoxicillin resistance in 12-week-old puppies has a prevalence more than double the next most common resistance, but at 16 weeks its prevalence is similar to that of resistance to other agents. This suggests that the causative factor for amoxicillin resistance early on is strong selection, rather than active selection against amoxicillin resistance later on as, resistance in general tends to reduce over time.

Table 4.5 Number of puppy faecal samples carrying *E. coli* resistant to any of the five different antibiotics (ciprofloxacin, tetracycline, amoxicillin, cephalexin and streptomycin) from the same puppies at 12 weeks and 16 weeks. *P*-values were calculated using Fisher's Exact Test.

	12-week-old puppies	**16-week-old puppies**	***p*-value**
Ciprofloxacin	5/41	2/41	0.43
Tetracycline	14/41	9/41	0.33
Amoxicilin	33/41	11/41	**<0.001**
Cephalexin	8/41	4/41	0.35
Streptomycin	12/41	6/41	0.18

The amount of resistance to the five antimicrobials in the *E. coli* obtained from the locally recruited 16-week-old puppy faecal samples was compared with that from locally recruited adult dog cohort samples (Table 4.6). There were no significant differences between resistance in the *E. coli* from the locally recruited 16-week-old puppy samples and locally recruited adult dog samples found (Table 4.6).

This could indicate that by 16-weeks-old puppies that are able to walk freely in public places and are exposed to the natural environment as well as other humans and animals have a wider variety of gut bacteria. This study shows that resistance at 16 weeks is similar to that seen in adult dogs. It may also indicate that the locally recruited 16-week-old puppies and locally recruited adult dogs have similar levels of resistance and this could be because they attend the same veterinary practices with similar antimicrobial prescribing policies and live in the same region (e.g. they walk in the same areas and are part of the same local dog population), so differences are not evident.

A significant difference was shown between the number of puppies with amoxicillin-resistant *E. coli* (higher at 12 weeks compared to the same puppies at 16 weeks; $p<0.001$; Table 4.5). It is possible that the high levels of resistance

seen in 12-week-old puppies (perhaps driven by maternal transmission) very quickly falls off after puppies start to interact with the environment and the puppies become more like the adult dog population in terms of resistance. However, another possible hypothesis to explain the relatively high levels of amoxicillin resistance seen in 12-week-old puppies could be that puppy owners are likely to acquire their puppy from a wide geographical area. Even the 'locally recruited' 12-week-old puppies may have come from a variety of places in the UK where amoxicillin resistance levels may be higher, as seen in the Generation Pup and Oxfordshire adult dogs (Table 4.3 and 4.4). This hypothesis would need to be investigated further as data on where the puppies originated from was not included in this study. Irrespective of the source of these high levels of amoxicillin resistance in 12-week-old puppies, by 16 weeks the resistance in *E. coli* carried by the puppies was more representative of the local area was lower than what was found in dogs from a wider geographical range (Table 4.5; Table 4.6). It was shown that the adult Oxfordshire dogs and the Generation Pup 16-week-old puppies had higher amoxicillin resistance compared to locally recruited dogs (Table 4.3 and 4.4).

Table 4.6 Resistance to five different antibiotics (ciprofloxacin, tetracycline, amoxicillin, cephalexin and streptomycin) in *E. coli* from 16-week-old puppies compared to adult locally recruited dogs. *P*-values were calculated using Fisher's Exact Test.

	Local 16-week-old puppies	**Local Adult dogs**	***p*-value**
Ciprofloxacin	2/41	2/14	0.27
Tetracycline	9/41	4/14	0.72
Amoxicilin	11/41	3/14	1.00
Cephalexin	4/41	3/14	0.35
Streptomycin	6/41	3/14	0.68

In conclusion, the comparison of resistance between dogs of different ages showed that amoxicillin resistance in *E. coli* was higher in 12-week-old puppies compared to 16-week-old puppies whereas there was no significant difference between resistance to any antibiotic between 16-week-old puppies and adult dogs. Further research could be conducted to investigate the impact of the domestic environment has on resistance in young puppies and whether there is any amplification of resistance in puppies due to being handled by humans. Furthermore, studies could compare veterinary practices with different antimicrobial prescription policies to evaluate whether these practices impact the amount of resistance found in the local dog population. Amoxicillin resistance in the *E. coli* carried by dogs was also found to be significantly lower in adult dogs compared to young puppies as well as lower in the local area when comparing the location of the recruitment of the dogs. Therefore, it would be useful to collect amoxicillin-resistant *E. coli* isolates and sequence the whole genome of the *E. coli* to more exactly determine resistance mechanisms.

Chapter 5 – Molecular Analysis

5.1 Introduction

Chapter 3 identified potential risk factors associated with AMR in the *E. coli* carried by 16-week-old puppies. The aim of the work in this chapter was to investigate the mechanisms responsible for this resistance. The 16-week-old puppy faecal samples with *E. coli* colonies that were cephalexin-resistant (Chapter 3) were first tested for cefotaxime (3rd generation cephalosporin) resistance. A series of multiplex PCRs were carried out on a number of the cefotaxime-resistant *E. coli* isolated from the 12-week-old, 16-week-old or adult dog samples to detect β-lactamase genes (carried out by Jacqueline Findlay).

The 16-week-old puppy cefotaxime- or ciprofloxacin-resistant *E. coli* were sent off for whole genome sequencing to elicit details about the specific *E. coli* including the ST, the presence of β-lactamase genes and other resistance genes. MLST was carried out on the cefotaxime-resistant and ciprofloxacin-resistant *E. coli* obtained from the 16-week-old puppy faecal samples (carried out by Jacqueline Findlay and Oliver Mounsey). It was not possible to carry this out on all of the cephalexin- and ciprofloxacin-resistant *E. coli.* The methods were carried out as described in Chapter 2.

5.2 Results and Discussion

E. coli from the 12-week-old puppies, 16-week-old puppies and adult dog samples that were cephalexin-resistant were tested for cefotaxime resistance and any resistant *E. coli* then had a series of multiplex PCRs carried out on them. The results from the PCR showed that it was possible, in some isolates, to identify the presence of known ESBL β-lactamase genes and the presence of

ampC which are known to confer cefotaxime resistance. It can be assumed that the mechanism for cefotaxime resistance in the *E. coli* with a negative result on PCR for known cefotaxime resistance genes would be AmpC hyperproduction due to chromosomal mutations in the *ampC* promoter. Of the faecal samples obtained from the 16-week-old puppy samples, 20 had *E. coli* that were resistant to cefotaxime. The number of the dogs and puppies with each gene were identified. For example, seven out of 20 16-week-old puppies were shown to have a Group 1 CTX-M gene (Table 5.1). CTX-M-1 is the most commonly found ESBL type in European livestock and the second most common type associated with clinical human isolates in some countries including France and Italy (Kjeldsen *et al.* 2015). Furthermore, CTX-M-15 has been described as one of the most important types as it is found in nearly all human and animal populations and environments all across the world and has rapidly spread across the UK since its first detection (Cantón *et al.* 2012). Both of these enzymes are from CTX-M Group 1. Plasmid-mediated AmpC β-lactamase carriage was also found in the cefotaxime-resistant *E. coli* from six of the 16-week-old puppies, the genes being $bla_{\text{CMY-2,}}$ and $bla_{\text{DHA-1}}$ (Table 5.1). CMY genes have been found in *E. coli* from sick and healthy dogs throughout the world, which suggests that these genes are common in dogs (Rocha-Gracia *et al.* 2015). It has been suggested that the high frequency of this gene in dogs is because of the spread of a few specific plasmids or the integration of this gene into many plasmids which has resulted in the spread of this plasmid throughout the UK dog population (Wedley *et al.* 2017). The PCR results in this study, however, show that puppies had a range of different genes detected their cefotaxime-resistant *E. coli.* Seven of the *E. coli* isolated from the 16-week-old puppies were found to be cefotaxime-resistant but had a negative result on PCR. A possible explanation for this could be that the mechanism for the *E. coli* to have cefotaxime resistance was chromosomal mutations leading to AmpC hyper-production.

Table 5.1 Results from the PCR showing the number of 12-week-old and 16-week-old puppies or adult dogs that have CTX-M Groups 1 or 9, OXA-1, CMY, DHA, or TEM. The *E. coli* tested were all cefotaxime-resistant.

	Number of 12-week-old puppy samples (n=6)	**Number of 16-week-old puppy samples (n=20)**	**Number of adult dog samples (n=2)**
CTX-M G1	1	7	1
CTX-M G9	2	1	0
***bla*$_{CMY}$**	4	4	1
***bla*$_{DHA}$**	0	1	0
***bla*$_{TEM}$**	2	2	0
Negative for these specific genes.	2	7	0

The cefotaxime-resistant *E. coli* from the 16-week-old puppy samples had whole genome sequencing carried out and the resulting data used to perform MLST. *E. coli* from samples obtained from puppies without completed questionnaires were excluded and those that were from 16-week-old puppies that were cefotaxime-resistant were included (n=20 *E. coli* colonies from 20 different puppies). This allowed identification of the ST to which the resistant *E. coli* belonged. Using a database, it was then possible to identify the other sources from which those particular ST has been found (not all *E. coli* in the database were necessarily resistant; *Enterobase* https://enterobase.warwick.ac.uk/*;* Table 5.2). This database does not show how commonly each ST is found in each source and only details that the specific STs has been found once before in that source. It was not possible to identify other places that ST6096 and ST2179 has been previously identified through the database (Table 5.2).

The molecular results from this chapter provide further information about AMR in *E. coli* from dogs and the possible acquisition of resistance through food, from other dogs and from other species and environments. These findings also

provide some evidence of the sharing of resistant *E. coli* between dogs and other species. There was a wide variety of different sequence types observed, with 16 different sequence types found (Table 5.2).

ST38, ST88 and ST10 have been reportedly been found in companion animals, livestock, wildlife and humans (Table 5.2; Ewers *et al.* 2012), indicating that some STs are widespread and found in many different animals and environments, including dogs. Many of the STs have been identified in dogs in the UK in the past. For example, ST10, ST963 and ST88 have all been reported in dogs in the UK (Wedley *et al.* 2017), suggesting that some STs are commonly found in dogs. Using *Enterobase*, it was possible to identify that ST372 and ST973 have been found in companion animals previously suggesting that these may be commonly found in dogs. Another study that carried out MLST on AMR dog samples also found *E. coli* with ST973, ST963 and ST372 (Melo *et al.* 2016), adding weight to the argument that these sequence types are found regularly in dogs.

In Chapter 3, analyses were carried out to assess whether there were risk factors that increased the risk of puppies carrying AMR *E. coli*. It was found puppies that rolled in cow pats had a greater risk of carrying *E. coli* with cephalexin resistance (Table 3.3). This indicates that agriculture - especially cattle - may contribute to the transmission of AMR to dogs. Three of the 16-week-old puppies had reportedly rolled in cow pats and had ST88, ST58 or ST3889; all of these sequence types have also been found in livestock (Tables 3.3 and 5.2). Therefore, this could suggest that the dogs are acquiring resistant *E. coli* from cow pats. However, ST88 is commonly found in companion animals, livestock, humans and wildlife, so more evidence will be needed to firmly link livestock to puppy *E. coli* (Ewers *et al.* 2012).

Of the 20 *E. coli* obtained from the puppy samples, 19 of STs found have been found previously in humans; this suggests that dogs and humans share many of the same STs. It is possible that *E. coli* is shared within a household among pets and humans. In fact, this host-to-host transmission has been found in many studies and this may facilitate the spread of AMR within the community (Johnson *et al.* 2008; Damborg *et al.* 2009; Grönthal *et al.* 2018). Research that evaluated the relationship of ESBL and AmpC production with multidrug resistant *E. coli*

isolated from clinical cases of canine urinary tract infection from 2002 to 2011 from the UK have also been compared to human samples from the same local area (Wagner *et al.* 2014). These authors also found ST372, ST10, ST744 and ST73 in the *E. coli* from the dog samples they obtained which were also found in this study (Table 5.2). However, these authors did not find enough evidence to suggest that there was a zoonotic spread of resistance between dogs and humans using ST results (Wagner *et al.* 2014). However, other studies have found evidence that dogs share similar strains of ESBL-producing genes which suggests household transmission (Baede *et al.* 2017).

Table 5.2 Sequence types found in cefotaxime-resistant *E. coli* from the 16-week-old puppy samples and possible other sources where the sequence type has been found before using *Enterobase*. The questionnaire data of whether these puppies were reported to have rolled in cowpats or were raw fed is also included as this was significant in the risk factor analysis (Yes, No, / indicates that this information was not available; Chapter 3).

16-week-old puppies	Sequence Type (ST)	Sources ST previously found	Roll in Cowpat?	Raw Fed?
1	6096		No	No
2	963	Human, Water, Livestock, Wild Animals	/	No
3	88	Human, Companion/domesticated Animals, Poultry, Bird, livestock	Yes	Yes
4	58	Human, livestock, domesticated animals, poultry	Yes	No
5	155	Human, Bovine, Animal, Companion Animal, Poultry	No	No
6	88	Human, Companion/domesticated Animals, Poultry, Bird, livestock	No	Yes
7	602	Poultry, Human, Livestock	No	No

16-week-old puppies	Sequence Type (ST)	Sources ST previously found	Roll in Cowpat?	Raw Fed?
8	10	Human, Companion Animal, Livestock	No	No
9	1196	Human, Environment, Poultry	No	No
10	215	Human, Poultry, Livestock, Environment	No	No
11	75	Companion Animal, Poultry, Livestock, Human, Environment	No	No
12	744	Human, Bird, Poultry, Livestock, Environment	No	No
13	3889	Animal, Companion Animal, Human, Poultry, Livestock	Yes	No
14	973	Poultry, Livestock, Human, Environment, Companion Animal, Wild Animal	No	No
15	372	Companion Animal. Domesticated Animal, Poultry, Human	No	No
16	744	Human, Bird, Poultry, Livestock, Environment	No	Yes
17	38	Human, Wild Animal, Poultry, Bird, Companion Animal	No	No
18	88	Human, Companion/ domesticated Animals, Poultry, Bird	No	No
19	88	Human, Companion/ domesticated Animals, Poultry, Bird	/	Yes
20	2179		/	Yes

There were 20 *E. coli* that were cefotaxime-resistant isolated from the 16-week-old puppies and these had their β-lactamase genes detected using whole genome sequencing (Table 5.3). The genes found indicate the type of resistance that the puppy carried and it is possible to identify whether some genes are more common than others. Four puppies were found to have bla_{CMY-2} and four were found to have $bla_{CTX-M-1}$; these were therefore the most commonly found genes detected in the cefotaxime-resistant *E. coli.* Three of the puppies were found to carry *E. coli* with $bla_{CTX-M-15}$ which is commonly found in humans (Cantón *et al.* 2012). The whole genome sequencing results matched the results found with PCR (Table 5.1; Table 5.3). Out of the 20 puppy *E. coli* samples, three had bla_{TEM-1B} or bla_{TEM-78} (Table 5.8). TEM-1 β-lactamases act by hydrolysing the β-lactam ring of antimicrobials and are found in both humans and animals across the world (Salverda *et al.* 2010).

The whole genome sequencing confirmed the PCR results - seven of the *E. coli* isolated from the 16-week-old puppies (three, four, five, six, seven, eleven and nineteen) have *ampC* promoter changes which caused AmpC hyperproduction that resulted in the destruction of the cefotaxime antimicrobial molecules, resulting in resistance (Table 5.3). A study examining prescribing at small animal veterinary practices in the UK found that clavulanic acid-potentiated amoxicillin was the most common antimicrobial prescribed (36%) and amoxicillin was the second most prescribed antimicrobial (20%; Radford *et al.* 2011). It could therefore be hypothesised that the dominance of AmpC found in the *E. coli* carried by dogs could be because of the high levels of usage of amoxicillin-type antimicrobials in the dog population. Nine of the 16-week-old puppies had ESBL genes (which are mediated by plasmids) detected. The rapid emergence and spread of ESBLs poses a serious health risk as multiple antimicrobials (such as third generation cephalosporins) used to treat infections caused by ESBL-producing bacteria are ineffective (Leonard *et al.* 2017).

Table 5.3 β-lactamase genes detected in the sequenced *E. coli*. The *E. coli* was obtained from 16-week-old puppies and were found to be cefotaxime-resistant. Genes detected include *bla*$_{CMY-2}$ and *bla*$_{CTX-M-15;}$ genes detected from each of these puppies is included.

16-week-old puppies	β-Lactamase Genes Detected							
	***bla*$_{TEM-78}$**	***bla*$_{TEM-1B}$**	***bla*$_{CMY-2}$**	***bla*$_{CTX-M-1}$**	***bla*$_{DHA-1}$**	***bla*$_{CTX-M-15}$**	***bla*$_{CTX-M-65}$**	***bla*$_{OXA-1}$**
1	Y		Y					
2			Y					Y
3								
4								
5								
6								
7								
8				Y				
9					Y			
10						Y		
11								
12				Y				
13		Y				Y		
14			Y					
15			Y					
16				Y				
17						Y		Y
18				Y				
19								
20		Y					Y	Y

From the sequencing of the cefotaxime-resistant *E. coli* isolated from the 16-week-old puppies, it was possible to detect other resistance genes which could potentially correspond to resistance to different antimicrobials. There was a wider range of AMR genes found in the puppy isolates - 25 different other resistance genes were found, including sulphonamides (11 puppies had *sul2,* two puppies had *sul1),* tetracycline resistance genes (nine puppies had *tet(B)* and four puppies had *tet(A),* as well as other resistance genes *floR, fosA7, mph(A), dfrA17, cat1A, catB4, dfrA1 and dfra14* (Table 5.4). This is a similar finding to a previous paper looking at resistance in adult dogs which found resistance genes like the ones found in this study and in this paper they stated that some of these resistance genes are also commonly identified in human isolates (Wedley *et al.* 2017). The resistance genes *dfrA1, dfrA17 and dfrA14* are responsible for trimethoprim resistance and were detected in five of the 16-week-old puppies and have been previously detected in other studies that have isolated *E. coli* and found them to be of animal origin (Wedley *et al.* 2011). The resistance genes *qnrB4, qnrS1, qnrS2* and *aac(6')Ib-c*r were detected in the *E. coli* isolated from the 16-week-old puppies (Table 4.7); these are fluoroquinolone resistance genes and give low level resistance to quinolones (Martinez-Martinez *et al.* 1998; Wedley *et al.* 2011).

It was possible in this study to predict whether the *E. coli* carried by the puppies were resistant to any other antibiotics using the sequencing data. These data suggested the potential for multidrug resistance (resistance to three or more classes of antimicrobials) in some of the puppies. Of the 20 cefotaxime-resistant *E. coli* colonies sequenced, 16 had resistance genes that potentially could cause resistance to three or more different classes of antimicrobials (Table 5.4). For example, the *E. coli* obtained from puppy 16 had resistance genes *sul2, tet(B), mph(A), dfrA17, catA1, aadA5, strA, strB* and aac(6')Ib-cr which confer resistance to seven different classes of antimicrobials (Table 5.4).

Table 5.4 Other resistance genes detected during the sequencing of cefotaxime-resistant *E. coli* obtained from 16-week-old puppies. For each of the puppies, the other resistance genes that were detected and the resistance that each gene corresponds to are shown. The puppies with *E. coli* that was potentially multidrug-resistant are also included.

Other resistance genes detected	**16-week-old puppy samples**																			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Sulphonamide resistance genes																				
sul1 - sulphonamide resistance gene		Y							Y											
sul2 – sulphonamide resistance gene	Y		Y	Y		Y		Y			Y	Y	Y			Y		Y	Y	
Tetracycline resistance gene																				
tet(A) – tetracycline resistance gene								Y		Y			Y				Y			
tet(B) – tetracycline resistance gene			Y	Y		Y					Y	Y	Y			Y		Y	Y	
Macrolide resistance genes																				
mph(A) – macrolide phophotansferases resistance gene									Y			Y				Y				
Fluoroquinolone Resistance																				

Other resistance genes detected	**16-week-old puppy samples**																			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Genes																				
qnrB4									Y											
qnrS1										Y			Y							
qnrS2																				Y
aac(6')Ib-cr																	Y			Y
Aminoglycosides Resistance Genes																				
aadA1- streptomycin, spectinomycin resistance gene		Y															Y			
aadA5- streptomycin, spectinomycin resistance gene												Y				Y				
strA – streptomycin resistance gene			Y	Y		Y		Y			Y	Y	Y			Y		Y	Y	
strB – streptomycin resistance gene			Y	Y		Y		Y			Y	Y	Y			Y		Y	Y	
aph(3')-Ia – aminoglycoside resistance gene			Y			Y						Y				Y			Y	
aac(6')Ib-cr – aminoglycoside resistance gene																	Y			Y
aac(3)-IIa - aminoglycoside resistance gene																	Y			
Other resistance																				

Other resistance genes detected	**16-week-old puppy samples**																			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
genes																				
floR – florfenicol resistance gene	Y					Y													Y	Y
fosA7 – fosfomycin resistance gene							Y				Y									
dfrA1 – trimethoprim resistance gene																	Y			
dfrA14 – trimethoprim resistance gene													Y							
dfrA17 – trimethoprim resistance gene									Y			Y				Y				
catA1 – chloramphenicol resistance gene												Y				Y				
catB4 – chloramphenicol resistance gene																	Y			
catB3 – chloramphenicol resistance gene																				Y
arr-3 – rifamycin resistance gene																				Y
Potentially Multidrug Resistant?	Y	Y	Y	Y		Y		Y	Y	Y	Y	Y	Y			Y	Y	Y	Y	Y

It was also possible to have the 16-week-old ciprofloxacin-resistant puppy *E. coli* whole genome sequenced. Twenty samples were sent off for sequencing and

the STs, β-lactamase genes and other resistance genes were detected.

There were 12 different STs identified from the 20 different 16-week-old puppies, indicating that there are a variety of different sequence types associated with ciprofloxacin-resistant *E. coli.* ST744 was the most common ST and was found in ten different puppies. This ST has previously been found in multidrug resistant *E. coli* obtained from dogs, suggesting that this ST is commonly found in dogs (Wagner *et al.* 2014). Many STs have been found in a wide variety of different animals and environments including humans, companion animals, wild animals, livestock, poultry and the environment which is evidence that many of the STs are found in many different places and not exclusively found in a particular species.

The *E. coli* isolated from the 16-week-old puppies that were ciprofloxacin-resistant were sequenced. Out of the 20 puppies whose ciprofloxacin resistant *E. coli* was sequenced, 12 were raw fed, and there were a variety of STs for these puppies (Table 5.5, Table 5.6). All of the raw-fed puppy sequence types have previously been found in poultry and birds which could be evidence that the 16-week-old puppies are acquiring resistant *E. coli* from eating raw poultry. It should be noted, however, that many of the STs are widespread and found in a variety of different species and environments. It is possible that the variety of different STs found in the ciprofloxacin-resistant *E. coli* from raw-fed puppies are the result of a variety of different STs in *E. coli* found in raw dog food, however, survey and testing of raw dog food would be required to establish this. Previous research has strongly suggested that feeding a dog raw food greatly increases the risk of the carriage of resistance, especially ciprofloxacin resistance (Schmidt *et al.* 2015; Wedley *et al.* 2017). This study presents yet more evidence that the puppies could be obtaining resistant *E. coli* from raw poultry meat as the STs identified are also found in poultry.

A previous study that investigated raw feeding in household cats also found that feeding raw food was a risk factor for ESBL shedding and that ESBL-producing *Enterobacteriacea* was often found to contaminate raw pet food (Baede *et al.* 2017). In this study the authors found that 77.8% of all investigated raw pet food was contaminated with viable ESBLs, yet none of the non-raw pet food was

contaminated with these bacteria (Baede *et al.* 2017). This provides further evidence that the raw food may be spreading resistance to companion animals.

Studies in the past have found that *E. coli* obtained from dogs and cats possess the same ESBL-encoding genes as well as the same STs as those from humans (Baede *et al.* 2017). The STs identified in the puppies in this study are found in a variety of different environments and animals, therefore further work may need to be done to assess whether the *E. coli* from the raw fed dogs originated from poultry. However, raw feeding dogs has been shown to be a risk factor for AMR and the ST results presented here provide further evidence for this (Chapter 3; Wedley *et al.* 2017). There is a possibility that dog owners may contaminate themselves with raw food whilst they are preparing their dogs' food and this may contribute to household transmission of AMR, hence it is important that owners are aware of this potential risk (Baede *et al.* 2017).

ST744 was found in seven *E. coli* obtained from 16-week-old puppies that were raw fed and was also found in three puppies that were not raw fed (Table 5.6). Some STs, however, were only found in puppies that were raw fed (ST1011, ST1196, ST1431, ST58, ST453, ST117, ST1775; Table 5.6). Some *E. coli* sequence types are more similar than others and using the seven MLST housekeeping genes it is possible to compare the relatedness of different sequence types (Lukjancenko *et al.* 2010). For example, ST744 and ST162 are fairly similar as 744 has *adk_10* and 162 has *adk_9* suggesting that these STs are more related than other STs (e.g. ST117 *adk_20;* Table 5.6). Both of these *E. coli* STs were commonly found in the puppies suggesting that they are common in dogs (Table 5.6). Therefore, the ST results may support the assertion that raw feeding is a risk factor for resistance, as the same STs have been in the puppies and previously found in poultry however, more work is needed.

The risk factor analysis in Chapter 3 showed that there are other potential risk factors associated with ciprofloxacin resistance, hence raw food does not explain all of the resistance found in the *E. coli* carried by the 16-week-old puppies. Playing in lake water was also found to be a risk factor for ciprofloxacin resistance (Chapter 3), however, only six of the 26 *E. coli* samples sent off for sequencing were obtained from puppies that played in lake water (Table 5.2). There was no

strong correlation found between puppies that swam in lake water and certain STs.

It was also possible to compare the STs in the cefotaxime-resistant *E. coli* carried by the 16-week-old puppies and the ciprofloxacin-resistant *E. coli.* ST58, ST1196 and ST744 were found in both the cefotaxime- and ciprofloxacin-resistant *E. coli* however, the other STs were only found in one or the other (Table 5.2 and Table 5.5).

Table 5.5 Sequence types found in the ciprofloxacin-resistant *E. coli* from the 16-week-old puppy samples including possible other sources where the sequence type has been found before using *Enterobase.* Results from the questionnaire data of whether these puppies were raw fed or whether they played in lake water is also included (Chapter 3).

16-week-old puppies	Sequence Type (ST)	Sources ST previously found	Raw fed?	Played in lake water?
18	7366	Human	No	No
19	162	Companion Animal, Human, Wild animal, Poultry, Domesticated Animal, Livestock, Environment, Food	Yes	No
16	744 1775	Human, Bird, Poultry, Livestock, Environment Poultry, Human	Yes	No
20	744	Human, Bird, Poultry, Livestock, Environment	Yes	No
21	162	Companion Animal, Human, Wild animal, Poultry, Domesticated Animal, Livestock, Environment, Food	No	No

16-week-old puppies	Sequence Type (ST)	Sources ST previously found	Raw fed?	Played in lake water?
22	162 117	Companion Animal, Human, Wild animal, Poultry, Domesticated Animal, Livestock, Environment, Food Bird, Human, Domesticated Animal, Poultry, Companion Animal, Water, Avian	Yes	Yes
13	744	Human, Bird, Poultry, Livestock, Environment	No	No
23	453 58 744	Human, Livestock, Poultry Human, livestock, domesticated animals, poultry Human, Bird, Poultry, Livestock, Environment	Yes	No
12	744 224	Human, Bird, Poultry, Livestock, Environment Poultry, Human, Domesticated Animal, Environment	No	Yes
24	162	Companion Animal, Human, Wild animal, Poultry, Domesticated Animal, Livestock, Environment, Food	Yes	No
25	744	Human, Bird, Poultry, Livestock, Environment	Yes	No
26	744	Human, Bird, Poultry, Livestock,	No	Yes

16-week-old puppies	Sequence Type (ST)	Sources ST previously found	Raw fed?	Played in lake water?
		Environment		
27	744	Human, Bird, Poultry, Livestock, Environment	Yes	No
28	744	Human, Bird, Poultry, Livestock, Environment	Yes	Yes
6	1431	Human, Food, Environment, Poultry, Aquatic	Yes	Yes
29	1196 1011	Human, Environment, Poultry Domesticated Animal, Human, Companion Animal, Food	Yes	No
30	4988	Human	No	No
31	162	Companion Animal, Human, Wild animal, Poultry, Domesticated Animal, Livestock, Environment, Food	No	No
32	Unknown 744	 Human, Bird, Poultry, Livestock, Environment	Yes	Yes

Table 5.6 Sequence types found in the ciprofloxacin-resistant *E. coli* from the 16-week-old puppy samples. Questionnaire data of whether these puppies were raw fed or not are included to show the number of times each sequence type is found in the *E. coli* carried by the dogs that are raw fed or non-raw fed (some puppies had more than one *E. coli* sequenced; Chapter 3).

Sequence Type and genes	**Raw fed (n=16)**	**Non-raw fed (n=7)**
744 *adk_10, fumc_11, gyrb_135, icd_8, mdh_8, pura_8, reca_2*	7	3
162 *adk_9, fumc_65, gyrb_5, icd_1, mdh_9, pura_13, reca_6*	3	2
4988 *adk_10, fumc_11, gyrb_421, icd_8, mdh_8, pura_8, reca_2*	0	1
1011 *adk_6, fumc_4, gyrb_159, icd_44, mdh_112, pura_1, reca_17*	1	0
1196 *adk_6, fumc_6, gyrb_33, icd_26, mdh_11, pura_8, reca_2*	1	0
1431 *adk_6, fumc_65, gyrb_3, icd_1, mdh_11, pura_13, reca_6*	1	0
224	0	1

Sequence Type and genes	**Raw fed (n=16)**	**Non-raw fed (n=7)**
adk_6, fumc_4, gyrb_33, icd_16, mdh_11, pura_8, reca_6		
58 *adk_6, fumc_4, gyrb_34, icd_16, mdh_24, pura_8, reca_14*	1	0
453 *adk_99, fumc_6, gyrb_33, icd_33, mdh_24, pura_8, reca_7*	1	0
117 *adk_20, fumc_45, gyrb_41, icd_43, mdh_5, pura_32, reca_2*	1	0
1775 *adk_6, fumc_31, gyrb_5, icd_28, mdh_1, pura_2, reca_2*	1	0
7366 *adk_112, fumc_11, gyrb_5, icd_12, mdh_8, pura_7, reca_86*	0	1

The ciprofloxacin resistant *E. coli* isolated from the 16-week-old puppies underwent whole genome sequencing which allowed the comparison of its ST with the resistance mechanism to assess for any correlation (Table 5.7). Mutational-driven resistance was more common than horizontal gene transfer in the ciprofloxacin-resistant *E. coli* and only three of the *E. coli* tested had resistance involving horizontal gene transfer. The results show that all the *E. coli* with ST 162 (*n*=5) had chromosomal mutations in DNA topoisomerase genes that confer ciprofloxacin resistance. For ST744, eight *E. coli* had chromosomal

mutations that conferred ciprofloxacin resistance and three *E. coli* had mobile genetic elements which conferred ciprofloxacin resistance (Table 5.7). The only other ST with horizontal gene transfer involved in the resistance mechanism was ST4988 (Table 5.7).

Table 5.7 Sequence types found in the ciprofloxacin-resistant *E. coli* from the 16-week-old puppy samples. The results from MLST show the mechanisms for resistance, whether resistance was likely to be mutational driven resistance or through horizontal gene transfer (Chapter 3).

Sequence Type and genes	**Mutational driven resistance, chromosomal mutation,**	**Horizontal gene transfer, mobile genetic element** ***qnr and aac-6'-CR***
744	8	2
162	5	0
4988	0	1
1011	1	0
1196	1	0
1431	1	0
224	1	0
58	1	0
453	1	0
117	1	0
1775	1	0
7366	1	0

Resistance genes were detected through the sequencing of the ciprofloxacin-resistant *E. coli* that was obtained from 16-week-old puppies (Table 5.8). There were 27 resistance genes detected (Table 5.8). Three of the ciprofloxacin resistant *E. coli* isolated from the 16-week-old puppies carried a *qnrS1* gene which confers fluoroquinolone resistance (Table 5.8). This plasmid-mediated

resistance occurs as the *qnr* gene may block the action of quinolones and usually confers low levels of resistance but may provide a background for the selection of additional chromosomal mutation-driven resistance (Fàbrega *et al.* 2009).

The remainder of the ciprofloxacin-resistant *E. coli* (n=19) carried by the puppies were likely to be due to chromosomal mutations through *gyrA* and *parC* mutations which confer AMR (Fàbrega *et al.* 2009; Table 5.8). Resistance occurs as a result of alterations in the target enzyme (DNA gyrase and topoisomerase IV) or because of a reduction of drug accumulation (Jacoby, 2009) This suggests that the majority of ciprofloxacin-resistant *E. coli* found in the 16-week-old puppies arose due to chromosomal mutation driven resistance (Table 5.7).

Of the 20 puppy *E. coli* sequenced, 16 carried the resistant gene *sul2* which confers resistance to sulphonamides. The spread of AMR has been attributed to class 1 and class 2 integrons and the *sul1* gene is normally linked to other resistance genes in class 1 integrons (Antunes *et al.* 2005), which may be a reason for the widespread of this gene in the ciprofloxacin-resistant *E. coli* obtained in this study. Resistance to multiple antimicrobials is associated with integrons which acquire and spread resistance genes. Sulphonamides are also known to have been used alone and in combination with trimethoprim to treat small animals (Chang *et al.* 2014).

The resistance gene *tet(B)* was also widespread and found in 13 of the 20 sequenced *E. coli.* This gene confers tetracycline resistance (Table 5.8). Tetracycline resistance genes have been commonly found in other studies in dogs and some have suggested this is due to the mobile nature of the resistance mechanism (Wedley *et al.* 2011). It has also been suggested that tetracyclines are regularly used on dogs, hence contributing to the amount of tetracycline resistance genes (Wedley *et al.* 2017).

No aminoglycoside resistance genes were detected in the ciprofloxacin-resistant *E. coli* and a possible explanation for this could be that aminoglycoside usage in dogs is low, therefore there are low levels of resistance (Radford *et al.* 2011; Table 5.8). Often, the mechanism for aminoglycoside resistance is via mobile genetic elements (Garneau-Tsodikova *et al.* 2016*)*.

Multidrug resistance is when more than three different classes of antibiotic

resistance can be detected in a bacterium. Out of the 19 ciprofloxacin-resistant *E. coli* sequenced, 18 had genes that confer resistance for three or more different antibiotic classes. Therefore, 18 out of 19 of the puppy *E. coli* tested were potentially multidrug resistant (Table 5.8).

Table 5.8 Resistance genes detected during the sequencing of the ciprofloxacin-resistant *E. coli* obtained from 16-week-old puppies.

Resistance genes detected	**18**	**17**	**19**	**20**	**21**	**13**	**22**	**12**	**23**	**24**	**25**	**26**	**27**	**6**	**28**	**29**	**30**	**31**	**32**
β-lactamase genes detected																			
bla$_{TEM-1c}$									Y										
bla$_{TEM-1B}$		Y	Y		Y	Y	Y	Y	Y		Y		Y	Y		Y		Y	Y
bla$_{CARB-2}$									Y										
bla$_{CTX-M-1}$								Y				Y							
bla$_{CTX-M-15}$																	Y		
Sulphonamide resistance genes																			
sul1 - sulphonamide resistance gene		Y		Y	Y	Y	Y	Y		Y		Y			Y			Y	
sul2 – sulphonamide resistance gene	Y	Y	Y	Y	Y	Y	Y	Y		Y	Y	Y		Y	Y	Y		Y	Y
sul3 – sulphonamide resistance gene							Y								Y				
Tetracycline resistance genes																			

Resistance genes detected	**18**	**17**	**19**	**20**	**21**	**13**	**22**	**12**	**23**	**24**	**25**	**26**	**27**	**6**	**28**	**29**	**30**	**31**	**32**
tet(A) – tetracycline resistance gene		Y		Y				Y						Y	Y	Y			Y
tet(B) – tetracycline resistance gene	Y	Y	Y		Y	Y	Y	Y		Y	Y	Y	Y				Y	Y	
tet(L) – tetracycline resistance gene													Y						
tet(M) – tetracycline resistance gene													Y						
Macrolide resistance genes																			
mph(A) – macrolide phophotansferases resistance gene		Y				Y		Y		Y	Y	Y						Y	
erm(B) – Erythromycin resistance gene													Y						
msr (C) - Erythromycin resistance gene													Y						
Fluoroquinolone resistance genes																			
qnrS1							Y							Y				Y	
Other resistance genes																			
lsa (E) – pleuromutilin – lincosamide – streptogramin resistance gene													Y						
dfrA1 – trimethoprim resistance gene		Y		Y	Y										Y			Y	

Resistance genes detected	**18**	**17**	**19**	**20**	**21**	**13**	**22**	**12**	**23**	**24**	**25**	**26**	**27**	**6**	**28**	**29**	**30**	**31**	**32**
dfrA5 – trimethoprim resistance gene							Y							Y					
dfrA7 – trimethoprim resistance gene								Y											
dfrA12 – trimethoprim resistance gene															Y				
dfrA14 – trimethoprim resistance gene																			Y
dfrA16 – trimethoprim resistance gene							Y												
dfrA17 – trimethoprim resistance gene	Y	Y				Y	Y	Y		Y	Y	Y						Y	
catA1 – chloramphenicol resistance gene	Y	Y		Y	Y	Y	Y	Y		Y	Y							Y	
cmlA1 – Chloramphenicol resistance gene							Y								Y				
floR – florfenicol resistance gene			Y											Y				Y	
Potentially multidrug resistant?	Y	Y	Y	Y	Y	Y	Y	Y		Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

In Chapters 3 and 4 it was found that amoxicillin resistance in the *E. coli* carried by 16-week-old puppies and adult dogs was lower in the locally recruited cohorts of dogs compared to the national cohorts of dogs (Chapter 3 and 4). Using the results in this chapter it was possible to compare the locally recruited 16-week-old puppies with the Generation Pup recruited puppies to see whether there were differences in the PCR and whole genome sequence results. Chi-squared tests

were carried out, however, no differences were detected between the local and national results for cefotaxime and ciprofloxacin resistance genes. However, in Chapters 3 and 4, it was amoxicillin resistance that showed the significant difference and this was not analysed in this chapter.

In conclusion, the sequence data provides further evidence of AMR in dogs. The STs found further implicate raw feeding of dogs and suggest a possible route of transmission as these STs have been found in both companion animals and poultry. The risk factor analysis in Chapter 3 highlighted some potential risk factors that contributed to AMR in *E. coli* carried by 16-week-old puppies and showed that raw feeding a puppy increased their risk of carrying AMR *E. coli*, however, it also showed that there were other associated risk factors. The genes found in the *E. coli* further reveal the mechanisms of resistance and that some resistance genes are found more commonly than others in the *E. coli* obtained from the dogs. As a result of this study, there is a set of 12-week-old, 16-week-old and adult dog faecal samples that can be used in future investigations. There is also a database of whole genome sequence results and PCR results from the *E. coli* obtained from the puppy and dog faecal samples that could be used to compare to other datasets including AMR in the environment, cattle, and humans. It may also be possible to whole genome sequence AMR *E. coli* found in raw dog food and compare these sequences to those found in the *E. coli* from the raw fed puppy samples.

In this chapter, only the 16-week-old puppy samples with *E. coli* that were cefotaxime- and ciprofloxacin-resistant had PCRs and were whole genome sequencing carried out. Further work could sequence the amoxicillin-resistant *E. coli* from the puppy samples to detect β-lactamase genes, determine the ST and provide evidence of the resistance mechanisms. In Chapters 3 and 4, amoxicillin resistance in the *E. coli* found in the puppies and dogs was shown to be significantly different in some of the various cohorts of dogs recruited, therefore this could provide evidence of transmission and acquisition of AMR *E. coli* in dogs and puppies. Further work could also be to survey the different types of raw food diet that dog owners feed to their dogs and then to whole genome sequence the *E. coli* found in various different types and brands of raw dog food to assess whether the STs and resistance genes are similar in the different types. It would

also be possible to compare the resistance genes, mechanisms and STs found in the dog *E. coli* with the *E. coli* found in the raw dog food.

References

Amos, G.C., Hawkey, P.M., Gaze, W.H., Wellington, E.M. 2014. Wastewater effluent contributes to the dissemination of CTX-M-15 in the natural environment. *Journal of Antimicrobial Chemotherapy.* **69**: 1785-1791.

Abernethy, J., Guy, R., Sheridan, E.A., Hopkins, S., Kiernan, M., Wilcox, M.H., Johnson, A.P., Hope, R, on behalf of the *E. coli* bacteraemia sentinel surveillance group. 2017. Epidemiology of *Escherichia coli* bacteraemia in England: results of an enhanced sentinel surveillance programme. *Journal of Hospital Infection.* **95**: 365-375.

Antunes, P., Machado, J., Sousa, J.C., Peixe, L. 2005. Dissemination of Sulfonamide Resistance Genes (*sul1, sul2 and sul3)* in Portuguese *Salmonella enterica* strains and relation with integrons. *Antimicrobial Agents and Chemotherapy.* **49**: 836-839.

Baede, V.O., Wagenaar, J.A., Broens, E.M., Duim, B., Dohmen, W., Nijsse, R., Timmerman, J.H. 2015. Longitudinal Study of Extended-Spectrum β-Lactamase and AmpC- Producing *Enterobacteriaceae* in Household Dogs. **59**: 3317-3124.

Baede, V.O., Broens, E.M., Spaninks, M.P., Timmerman, A.J., Graveland, H., Wagenaar, J.A., Duim, B., Hordijk. 2017. Raw pet food as a risk factor for shedding of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in household cats. *PLOS ONE.* 1-11.

Bloom, G., Merrett, G.B., Wilkinson, A., Lin, V., Paulin, S. 2017. Antimicrobial resistance and universal health coverage. *BMJ Global Health.* **2**: 1-6.

Blount, Z.D. 2015. The unexhausted potential of *E. coli. E.Life. 1-12.*

Bennett, P.M., Livesey, C.T., Nathwani, D., Reeves, D.S., Saunders, J.R., Wise, R. 2004. An Assessment of the risks associated with the use of antibiotic resistance genes in genetically modifies plants: report of the Working Party of the British Society for Antimicrobial Chemotherapy. *Journal of Antimicrobial Chemotherapy.* **53**: 418-431.

Bennett, P.M. 2008. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology.* **153**: 5347-5357.

Bonnet, R. 2004. Growing Group of Extended-Spectrum β-Lactamases: the CTX-M Enzymes. *Antimicrobial Agents and Chemotherapy.* **48**: 1-14.

Buckland, E.L., O'Neill, D., Summers, J., Mateus, A., Church, D., Redmond, L., Brodbelt, D. 2016. Characterisation of antimicrobial usage in cats and dogs attending UK primary care companion animal veterinary practices. *Veterinary Record.* **179**: 489.

Burmeister, A.R. 2015. Horizontal Gene Transfer. *Evolution, Medicine and Public Health.* 193-194.

Cantón, R., González-Alba, J.M., Galán, C. 2012. CTX-M enzymes: origin and diffusion, *Frontiers in Microbiology.* **3**: 1-19.

Carattoli, A., Lovari, S., Franco, A., Cordaro, G., Di Matteo, P., Battisti, A. 2005.

Extended-Spectrum β-Lactamases in *Escherichia coli* Isolated from Dogs and Rats in Rome, Italy, from 2001 to 2003. *Antimicrobial Agents and Chemotherapy.* **49**: 833-835.

Carvalho, A.C., Barbosa, A.V., Arais, L.R., Ribeiro, P.F., Carneiro, V.C., Cerqueira, A.M.F. 2016. Resistance patterns, ESBL genes, and genetic relatedness of *Escherichia coli* from dogs and owners. *Brazilian Journal of Microbiology.* **47**: 150-158.

Chang, S., Lo, D., Wei, H., Kuo, H. 2014. Antimicrobial resistance of *Escherichia coli* isolates from canine urinary tract infections. *The Journal of Veterinary Medical Science.* **77**: 59-65.

Chiou, J., Li, R., Chen, S. 2015. CARB-17 Family of β-Lactamases Mediates Intrinsic Resistance to Penicillins in *Vibrio parahaemolyticus.* **59**: 3593-3595.

Coleman., B.L., Salvadori, M.I., McGeer, A.J., Sibley, K.A., Neumann, N.F., Bondy, S.J., Gutmanis, I.A., McEwen, S.A., Lavoie, M., Strong, D., Johnson, I., Jamieson, F.B., Louie, M., ARO Water Study Group. 2012. The role of drinking water in the transmission of antimicrobial resistant *E. coli.* Epidemiology Infection. **140**: 633-642.

Costa, D., Poeta, P., Sáenz, Y., Coelho, A.C., Matos, M., Vinué, L., Rodrigues, J., Torres, C. 2007. Prevalence of antimicrobial resistance and resistance genes in faecal *Escherichia coli* isolates recovered from healthy pets. *Veterinary Microbiology.* **127**: 97-105.

Costa, D., Vinué, L., Poeta, P., Coelho, A.C., Matos, M., Sáenz, Y., Somalo, S., Zarazaga, M., Rodrigues, J., Torres, C. 2009. Prevalence of extended-spectrum

beta-lactamase-producing *Escherichia coli* isolates in faecal samples of broilers. *Veterinary Microbiology.* **138**: 339-344.

Damborg, P., Nielson, S.S., Guardabassi, L. 2009. Escherichia coli Shedding Patterns in Humans and Dogs: Insights into Within-Household Transmission of Phylotypes Associated with Urinary Tract Infections. *Epidemiology and Infection.* **137**: 1457-1464.

Davies, S.C., Catchpole, M., Tomkins, S., Cleary, P., Kessel, A., Wilson, J.C. 2011. Annual report of the chief medical officer. *Infection and the Rise of Antimicrobial Resistance.* **2.** Department of Health, London.

Day, M.J., Horzinek, M.C.,Schultz, R.D., Squires, R.A. 2016. Guidelines for the vaccination of dogs and cats. *Journal of Small Animal Practice.* **57**: 1- 45.

deKraker, M.E.A., Stewardson, A.J., Harbarth, S. 2016. Will 10 million people die a year due to antimicrobial resistance by 2050? *PLOS Medicine*. 1-6.

Ewers, C., Bethe, A., Semmler, T., Guenther, S., Wieler, L.H. 2012. Extended-spectrum-β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. **18**: 646-655.

Fàbrega, A., Madurga, S., Giralt, E., Vila, J. 2009. Mechanism of action of and resistance to quinolones. *Microbial Biotechnology.* **2:** 40-61.

Ferri, M., Ranucci, E., Romagnoli, P., Giaccone, V. 2015. Antimicrobial resistance: A global emerging threat to public health systems. *Critical reviews in*

food science and nutrition. **57:** 2857-2876.

Friedman, N.D., Temkin, E., Carmeli, Y. 2015. The negative impact of antibiotic resistance. *Clinical Microbiology and Infection.* **22**: 416-422.

Frost, I., Smith, W.P.J., Mitri, S., Millan, A.S., Davit, Y., Osborne, J.M., Pitt-Francis, J.M., MacLean, R.C., Foster, K.R. 2018. Cooperation, competition and antibiotic resistance in bacterial colonies. *The International Society for Microbial Ecology.* **12:** 1582-1593.

Gandolfi,P., Petrini, O., Ruggeri-Bernardi, N., Schelling, E. 2013. Extended-spectrum-β-lactamase- producing *Enterobacteriaceae* in healthy companion animals living in nursing homes and in the community. *American Journal of Infection Control.* **41**: 831-835.

Garibyan, L & Avashia, N. 2013. Research techniques made simple: Polymerase Chain Reaction (PCR). *The Journal of Investigative Dermatology.* **3**: 1-8.

Garneau-Tsodikova, S., Labby, K.J. 2016. Mechanisms of resistance to aminoglycoside antibiotics: overview and perspectives. *MedChemComm.* **7**: 11-27.

Gibson, J.S., Morton, J.M., Cobbold, R.N., Flippich, L.J., Trott, D.J. 2011. Risk factors for multi-drug resistant *Escherichia coli* rectal colonization of dogs on admission to a veterinary hospital. *Epidemiology and Infection.* **139**: 197-205.

Giedraitene, A., Vitkauskienė, A., Naginiene, E., Pavilonis, A. 2011. Antibiotic Resistance Mechanisms of Clinically Important Bacteria. *Medicina.* **47**: 137-146.

Guardabassi, L., Schwarz, S., Lloyd, D.H. 2004. Pet animals as reservoirs of antimicrobial-resistant bacteria. *Journal of Antimicrobial Chemotherapy.* **54**: 321-332.

Grönthal, T., Österlad, M., Eklund, M., Jalava, J., Nykäsenoja, S., Pekkanen, K., Rantala, M. 2018. Sharing more than friendship – transmission of NDM-5 ST167 and CTX-M-9 ST69 *Escherichia coli* between dogs and humans in a family, Finland, 2015. *Eurosurveillance.* **23:**1-10.

Grønvold, A.R., L'Abée-Lund, T.M., Sørum, H., Skancke, E., Yannarell, A.C., Mackie, R.I. 2010. Changes in fecal microbiota of healthy dogs administered amoxicillin. *FEMS Microbiology Ecology.* **71**: 313-326.

Harada, K., Morimoto, E., Kataoka, Y., Takahashi, T. 2011. Clonal spread of antimicrobial-resistant *Escherichia coli* isolates among pups in two kennels. *Acta Veterinaria Scandinavica.* **53**: 1-7.

Hockenhull, J., Turner, A.E., Reyher, K.K., Barett, D.C., Jones, L., Hinchliffe, S., Buller, H.J., 2017. Antimicrobial use in food-producing animals: a rapid evidence assessment of stakeholder practices and beliefs. *Vet Record.* **181**: 1-8.

Holmes, A.H., Moore, L.S.P., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., Guerin, P.J., Piddock, L.J.V. 2016. Understanding the mechanisms and drivers of antimicrobial resistance. *The Lancet.* **387**: 176-187.

Hordijk, J., Schoormans, A., Kwakemaak, M., Duim, B., Broens, E., Dierikx, C., Mevius, D., Wagenaar, J.A. 2013. High prevalence of fecal carriage of extended-

spectrum-β-lactamase/ AmpC- producing *Enterobacteriaceae* in cats and dogs. *Frontiers in Microbiology.* **4**: 1-5.

Hughes, L.A., Williams, N., Clegg, P., Callaby, R., Nuttall, T., Coyne, K., Pinchbeck, G., Dawson, S. 2012. Cross-sectional survey of antimicrobial prescribing patterns in UK small animal veterinary practice. *Preventive Veterinary Medicine.* **104:** 309-316.

Hutton, R. 2018. Antibiotic resistance in small animal veterinary practice: veterinary nurses as antibiotic guardians. *The Veterinary Nurse.* **9**: 4-10.

Ingti, B., Paul, D., Maurya, A.P., Bora, D., Chanda, D.D., Chakravarty, A., Bhattacharjee, A. 2017. Occurrence of *bla*$_{DHA-1}$ mediated cephalosporin resistance in *Escherichia coli* and their transcriptional response against cephalosporin stress a report from India. *Annals of Clinical Microbiology and Antimicrobials.* **16**: 1-8.

Jacoby, G.A. 2009. AmpC β-lactamases. *Clinical Microbiology Reviews.* **22**: 161-182.

Johnson, J.R., Clabots, C., Kuskowski, M.A. 2008. Multiple- Host Sharing, Long-Term Persistence, and Virulence of *Escherichia coli* Clones from Humans and Animals Household Members. *Journal of Clinical Microbiology.* **46**: 4078 - 4082.

Kjeldsen, T.S.B., Overgaard, M., Nielsen, S.S., Bortolaia, V., Jelsbak, L., Sommer, M., Guardabassi, L., Olsen, J.E. 2015. CTX-M-1 β-lactamase expression in *Escherichia coli* is dependent on cefotaxime concentration, growth phase and gene location. *Journal of Antimicrobial Chemotherapy.* **70**: 62-70.

Lau, S.H., Reddy, S., Cheesbrough, J., Bolton, F.J., Willshaw, G., Cheasty, T., Fox, A.J., Upton, M. 2008. Major uropathogenic *Escherichia coli* strain isolated in the Northwest of England identified by multilocus sequence typing. *Journal of Clinical Microbiology.* **46**: 1076-1080.

Leite-Martins, L.R., Mahú, M.I.M., Cost, A.L., Mendes, A., Lopes, E., Mendonça, D.M.V., Niza-Ribeiro, J.J.R., de Matos, A.J.F., da Costa, P.M. 2014. Prevalence of antimicrobial resistance in enteric *Escherichia coli* from domestic pets and assessment of associated risk markers using a generalized linear mixed model. *Preventative Veterinary Medicine.* **117**: 28-39.

Leonard, A.F.C., Zhang, L., Balfour, A.J., Garside, R., Hawkey, P.M., Murray, A.K., Ukoumunne, O.C., Gaze, W. 2017. Exposure to and colonisation by antibiotic-resistant *E. coli* in UK coastal water users: Environmental surveillance, exposure assessment, and epidemiological study (Beach Bum Survey). Environment International. 1-8.

Liu, C.M., Stegger, M., Aziz, M., Johnson, T.J., Waits, K., Nordstrom, L., Gauld, L., Weaver, B., Rolland, D., Stratham, S., Horwinski, J., Sariya, S., Davis, G.S., Sokurenko, E., Keim, P., Johnson, J.R., Price, L.B. 2018. *Escherichia coli* ST131-*H22* as a Foodborne Uropathogen. *American Society for Microbiology.* **9**: 1-11.

Lukjancenko, O., Wassenaar, T.M., Ussery, D.W. 2010. Comparison of 61 sequenced *Echerichia coli* genomes. *Microbiology Ecology.* **60**: 708-720.

Machado, E., Coque, T.M., Cantón, R., Sousa, J.C., Peixe, L. 2008. Antibiotic resistance integrons and extended-spectrum β-lactamases among Enterobacteriaceae isolates recovered from chickens and swine in Portugal.

Journal of Antimicrobial Chemotherapy. **62**: 296-302.

Magouras, I., Carmo, L.P., Stärk, K.D.C., Schüpbach-Regula, G. 2017. Antimicrobial Usage and Resistance in Livestock: Where Should We Focus? *Frontiers in Veterinary Science.* **4**: 1-4.

Martinez-martinez, L., Pascual, A., Jacoby, G.A. 1998. Quinolone resistance from a transferable plasmid. *The Lancet.* **351**: 797-799.

McNulty, C.A.M., Lecky, D.M., Xu-McCrae, L., Nakiboneka-Ssenabulya, D., Chung, K., Nichols, T., Thomas, H.L., Thomas, M., Alvarez-Buylla, A., Turner, K., Shabir, S., Manzoor, S., Smith, S., Crocker, L., Hawkey, P.M. 2018. CTX-M ESBL-producing Enterobacteriaceae: estimated prevalence in adults in England in 2014. *Journal of Antimicrobial Chemotherapy.* **73**: 1368-1388.

2, L.C., Boisson, M.N.G., Saras, E., Médaille, C., Boulouis, H., Madec, J., Haenni, M. 2016. OXA-48-producing ST372 *Escherichia coli* in a French Dog. *Journal of Antimicrobial Chemotherapy.* 1256-1258.

Milani, C., Corrò, M., Drigo, M., Rota, A. 2012. Antimicrobial resistance in bacteria from breeding dogs housed in kennels with differing neonatal mortality and use of antibiotics. *Theriogenology.* **78**: 1321-1328.

Mora, A., Herrera, A., Mamani, R., López, C., Alonso, M.P., Blanco, J.E., Blanco, M., Dahbi, G., García-Garrote, F., Pita, J.M., Coira, A., Bernárdez., Blanco, J. 2010. Recent Emergence of Clonal Group O25b:K1:H4:B2:ST131 *ibe*A Strains among *Escherichia coli* Poultry Isolates, Including CTX-M-9- Producing Strains, and Comparison with Clinical Human Isolates. *Applied and Environmental Microbiology.* **76**: 6991-6997.

Munita, J.M., Arias, C.A. 2016. Mechanisms of antibiotic resistance. *Microbiology Spectrum.* **4**: 1-37.

Murphy, C., Reid-Smith, R.J., Prescott, J.F., Bonnett, B.N., Poppe, C., Boerlin, P., Weese, J.S., Janecko, N., McEwen, S.A. 2009. Occurrence of antimicrobial resistant bacteria in healthy dogs and cats presented to private veterinary hospitals in southern Ontario: A preliminary study. *Canadian Veterinary Journal.* **50**: 1047-1053.

Naseer, U., Haldorsen, B., Simonsen, G.S., Sundsfjord, A. 2009. Sporadic occurrence of CMY-2 producing multidrug-resistant *Escherichia coli* of ST-complexes 38 and 448, and ST131 in Norway. *European Society of Clinical Microbiology and Infectious Diseases.* **16**: 171-178.

Normand, E.H., Gibson, N.R., Reid, S.W.J., Carmichael, S., Taylor, D.J. 2000. Antimicrobial-resistance trends in bacterial isolates from companion-animal community practice in the UK. *Preventive Veterinary Medicine.* **46**: 267-278.

Ogeer-Gyles, J., Matthews, K.A., Sears, W., Prescott, J.F., Weese, J.S., Boerlin, P. 2006. Development of antimicrobial drug resistance in rectal *Escherichia coli* isolates from dogs hospitalized in an intensive care unit. *Journal of the American Veterinary Medical Association.* **229**: 694-699.

Overdevest, I., Willemsen, I., Rijnsburger, M., Eustace, A., Xu, L., Hawkey, P., Heck, M., Savelkoul, P., Vandenbroucke-Grauls, C., van der Zwaluw, K., Huijsdens, X., Kluytmans, J. 2011. Extended-Spectrum-β-Lactamase Genes of *Escherichia coli* in Chicken Meat and Humans, the Netherlands. *Emerging Infectious Diseases.* **17**: 1216-1222.

Pavez, M., Neves, P., Dropa, M., Matté, M.H., Grinbaum, R.S., de Araújo, M.R.E., Mamizuka, E.M., Lincopan, N. 2008. Emergence of carbapenem-resistant *Escherchia coli* producing CMY-2 type AmpC β-lactamase in Brazil. *Journal of Medical Microbiology.* **57**: 1590-1592.

Pérez-Pérez, F.J., Hanson, N.D. 2002. Detection of Plasmid-Mediated AmpC β-Lactamase Genes in Clinical Isolates by Using Multiplex PCR. *American Society for Microbiology.* **40**: 2153-2162.

Prescott, J.F., Boerlin, P. 2016. Antimicrobial use in companion animals and Good Stewardship Practice. *Veterinary Record.* **179**: 486-488.

Radford, A.D., Noble, P.J., Coyne, K.P., Gaskell, R.M., Jones, P.H., Bryan, J.G.E., Setzkorn, C., Tierney, Á, Dawson, S. 2011. Antibacterial prescribing patterns in small animal veterinary practice identified via SAVSNET: the small animal veterinary surveillance network. *Veterinary Record.***169:** 1-8

Randall, L.P., Clouting, C., Horton, R.A., Coldham, N.G., Wu, G., Clifton-Hadley, F.A., Davies, R.H., Teale, C.J. 2011. Prevalence of *Escherichia coli* carrying extended spectrum β-lactamases (CTX-M and TEM-52) from broiler chickens and turkeys in Great Britain Between 2006-2009. **66**: 86-95.

Rocha-Gracia, R.C., Cortés-Cortés, G., Lozano-Zarain, P., Bello, F., Martínez-Laguna, Y., Torres, C. 2015. Faecal *Escherichia coli* isolates from healthy dogs harbour CTX-M-15 and CMY-2 β-lactamases. *The Veterinary Journal.* **3***:* 315-319.

RUMA. 2012. Antimicrobial use in veterinary medicine. *European Commission.*

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science.* **239**: 487-491.

Salverda, M.L.M., de Visser, J.A.G.M., Barlow, M. 2010. Natural evolution of TEM-1 β-Lactamase: experimental reconstruction and clinical relevance. *Federation of European Microbiological Societies.* **34**: 1015-1036.

Schaufler, K., Semmler, T., Wieler, L.H., Wöhrmann, M., Baddam, R., Ahmed, N., Müller, K., Kola, A., Fruth, A., Ewers, C., Guenther, S. 2015. Clonal spread and interspecies transmission of clinically relevant ESBL-producing *Escherichia coli* of ST410 – another pandemic clone? *FEMS Microbiology Ecology.* **92**: 1-9.

Schmidt, V.M., Pinchbeck, G.L., Nuttall, T., McEwan, N., Dawson, S., Williams, N.J. 2015. Antimicrobial resistance risk factors and characterization of faecal *E. coli* isolated from healthy Labrador retrievers in the United Kingdom. *Preventative Veterinary Medicine.* **119**: 31-40.

Shaikh, S., Fatima, J., Shakil, S., Mohd, S., Rizvi, D., Kamal, M.A. 2014. Antibiotic resistant and extended spectrum beta-lactamases: Types, epidemiology and treatment. *Saudi Journal of Biological Sciences.* **22**: 90-101.

Sidjabat, H.E., Townsend, K.M., Hanson, N.D., Bell, J.M., Stokes, H.W., Gobius, K.S., Moss, S.M., Trott, D.T. 2006. Identification of *bla*$_{CMY-7}$ and associated plasmid-mediated resistance genes in multidrug-resistant *Escherichia coli* isolated from dogs at a veterinary teaching hospital in Australia. **57**: 840-848.

Singleton, D.A., Sánchez-Vizcaíno, F., Dawson, S., Jones, P.H., Noble, P.J.M., Pinchbeck, G.L., Williams, N.J., Radford, A.D. 2017. Patterns of antimicrobial agent prescription in a sentinel population of canine and feline veterinary practices in the United Kingdom. *The Veterinary Journal.* **224**: 18-24.

Sirijatuphat, R., Sripanidkulchai, K., Boonyasiri, A., Rattanaumpawan, P., Supapueng, O., Kiratisin, P., Thamlikitkul, V. 2017. Implementation of global antimicrobial resistance surveillance system (GLASS) in patients with bacteremia. *PLOS one.* 1-15.

Stevens, K.B., Gilbert, J., Strachan, W.D., Robertson, J., Johnstone, A.M., Pfeiffer, D.U. 2007. Characteristics of commercial pig farms in Great Britain and their use of antimicrobials. *Veterinary Record.* **161**: 45-52.

Sulton, D., Pagan-Rodriguez, D., Zhou, X., Liu, Y., Hujer, A.M., Bethel, C.R., Helfand, M.S., Thomson, J.M., Anderson, V.E., Buynak, J.D., Ng, L.M., Bonomo, R.A. 2005. Clavulanic Acid Inactivation of SHV-1 and the Inhibitor-resistant S130G SHV-1 β-Lactamase. *The Journal of Biological Chemistry.* **280**: 35528-35536.

Tacconelli, E., Sifakis, F., Harbarth, S, Schrijver, R., van Mourik, M., Voss, A., Sharland, M., Rajendran, N.B., Rodriguez-Baño, J. 2017. Surveillance for control of antimicrobial resistance. *Lancet Infectious Diseases.* **18**: 99-106.

Tang, K.L., Caffrey, N.P., Nóbrega, D.B., Cork, S.C., Ronksley, P.E., Barkema, H.W., Polachek, A.J., Ganshorn, H., Sharma, N., Kellner, J.D., Ghali, W.A. 2017. Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a

systematic review and meta-analysis. *Lancet Planet Health.* **1**: 316-327.

Tenover, F.C 2006. Mechanisms of antimicrobial resistance in bacteria. *American Journal of Medicine.* **119**: 62-70.

Timofte, D., Maciuca, I.E., Williams, N.J., Wattret, A., Schmidt, V. 2016. Veterinary Hospital Dissemination of CTX-M-15 Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli* ST410 in the United Kingdom. *Microbial Drug Resistance.* **22**: 609-615.

Trott, D.J., Filippich, L.J., Bensink, J.C., Downs, M.T., McKenzie, S.E., Townsend, K.M., Moss, S.M., Chin, J.J. 2004. Canine model for investigating the impact of oral enrofloxacin on commensal coliforms and colonisation with multidrug-resistant *Eascherichia coli. Journal of Medical Microbiology.* **53**: 439-443.

van Hoek, A.H., Mevius, D., Guerra, B., Mullany, P., Roberts, A.P., Aarts, H.J. 2011. Acquired antibiotic resistance genes: an overview. *Frontiers in Microbiology.* **2:** 1-27.

Ventola, C.L. 2015. The Antibiotic Resistance Crisis. *Pharmacy and Therapeutics.* **40**: 277-283.

Wagner, S., Gally, D.L., Argyle, S.A. 2014. Multidrug resistant *Escherichia coli* from canine urinary tract infections tend to have commensal phylotypes, lower prevalence of virulence determinants and *ampC*-replicons. *Veterinary Microbiology.* **169**: 171-178.

Warren, R.E., Ensor, V.M., O'Neill, P., Butler, V., Taylor, J., Nye, K., Harvey, M., Livermore, D.M., Woodford, N., Hawkey, P.M. 2007. Imported chicken meat as a potential source of quinolone-resistant *Escherichia coli* producing extended spectrum β-lactamases in the UK. *Journal of Antimicrobial Chemotherapy.* **61**: 504-508.

Wedley, A.L., Maddox, T.W., Westgarth, C., Coyne, K.P., Pinchbeck, G.L., Williams, N.J., Dawson, S. 2011. Prevalence of antimicrobial-resistant *Escherichia coli* in dogs in a cross-sectional, community-based study. *Veterinary Record.* **168**.

Wedley, A.L., Dawson, S., Maddox, T.W., Coyne, K.P., Pinchbeck, G.L., Clegg, P., Nuttall, T., Kirchner, M., Williams, N.J. 2017. Carriage of antimicrobial resistant *Escherichia coli* in dogs: Prevalence, associated risk factors and molecular characteristics. *Veterinary Microbiology.* **199**: 23-30.

Woolhouse, M., Ward, M., van Bunnik, B., Farrar, J. 2015. Antimicrobial resistance in humans, livestock and the wider environment. *Philosophical Transactions, Royal Society.* **370**: 1-7.

Zurfluh, K., Glier, M., Hächler, H., Stephan, R. 2015. Replicon typing of plasmids carrying $bla_{CTX\text{-}M\text{-}15}$ among *Enterobacteriaceae* isolated at the environment, livestock and human interface. *Science of the Total Environment.* **521-522**: 75-78.

Zurfluh, K., Jakobi, G., Stephan, R., Hächler, H., Nüesch-Inderbinen, M. 2014. Replicon typing of plasmids carrying $bla_{CTX\text{-}M\text{-}1}$ in *Enterobacteriaceae* of animal, environmental and human origin. *Frontiers of Microbiology.* **5**: 1-5.

BSAVA Guide to the Use of Veterinary Medicines. 2018.

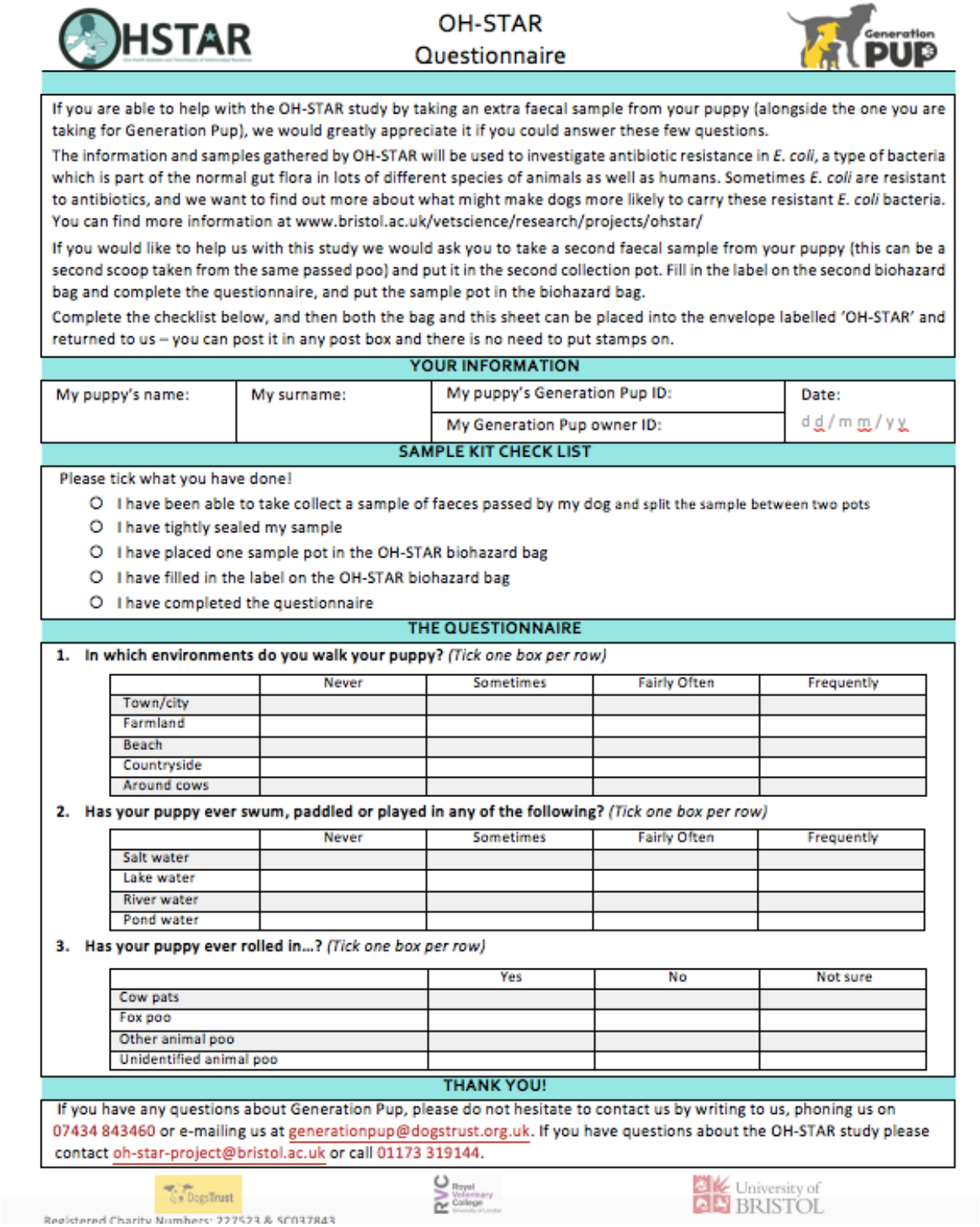
WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR). 2011. Critically Important Antimicrobials for Human Medicine. **3**: 1-32.

WHO Antimicrobial Resistance Global Report on Surveillance. 2014.

WHO list of Critically Important antimicrobials for human medicine. 5th Edition. 2017.

European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 8.0. 2018.

Appendix



OH-STAR Questionnaire

If you are able to help with the OH-STAR study by taking an extra faecal sample from your puppy (alongside the one you are taking for Generation Pup), we would greatly appreciate it if you could answer these few questions.

The information and samples gathered by OH-STAR will be used to investigate antibiotic resistance in *E. coli*, a type of bacteria which is part of the normal gut flora in lots of different species of animals as well as humans. Sometimes *E. coli* are resistant to antibiotics, and we want to find out more about what might make dogs more likely to carry these resistant *E. coli* bacteria. You can find more information at www.bristol.ac.uk/vetscience/research/projects/ohstar/

If you would like to help us with this study we would ask you to take a second faecal sample from your puppy (this can be a second scoop taken from the same passed poo) and put it in the second collection pot. Fill in the label on the second biohazard bag and complete the questionnaire, and put the sample pot in the biohazard bag.

Complete the checklist below, and then both the bag and this sheet can be placed into the envelope labelled 'OH-STAR' and returned to us – you can post it in any post box and there is no need to put stamps on.

YOUR INFORMATION

My puppy's name:	My surname:	My puppy's Generation Pup ID:	Date:
		My Generation Pup owner ID:	d d / m m / y y

SAMPLE KIT CHECK LIST

Please tick what you have done!

- ○ I have been able to take collect a sample of faeces passed by my dog and split the sample between two pots
- ○ I have tightly sealed my sample
- ○ I have placed one sample pot in the OH-STAR biohazard bag
- ○ I have filled in the label on the OH-STAR biohazard bag
- ○ I have completed the questionnaire

THE QUESTIONNAIRE

1. **In which environments do you walk your puppy?** *(Tick one box per row)*

	Never	Sometimes	Fairly Often	Frequently
Town/city				
Farmland				
Beach				
Countryside				
Around cows				

2. **Has your puppy ever swum, paddled or played in any of the following?** *(Tick one box per row)*

	Never	Sometimes	Fairly Often	Frequently
Salt water				
Lake water				
River water				
Pond water				

3. **Has your puppy ever rolled in…?** *(Tick one box per row)*

	Yes	No	Not sure
Cow pats			
Fox poo			
Other animal poo			
Unidentified animal poo			

THANK YOU!

If you have any questions about Generation Pup, please do not hesitate to contact us by writing to us, phoning us on 07434 843460 or e-mailing us at generationpup@dogstrust.org.uk. If you have questions about the OH-STAR study please contact oh-star-project@bristol.ac.uk or call 01173 319144.

Registered Charity Numbers: 227523 & SC037843

Figure 1. The questionnaire provided to 16-week-old puppy owners via Generation Pup. The questionnaire was completed and sent alongside a faecal sample collected from their puppy.

University of BRISTOL HSTAR

For office use only

Questionnaire 1

An investigation into carriage of antimicrobial resistant *E. coli* in young puppies

1. Your name_____

2. Your postcode_____

3. Your puppy's name_____

4. Your puppy's age and/or date of birth_____

5. Your puppy's breed_____

6. Which types of food has your puppy been fed? Please tick all that apply.

Commercial wet food (e.g. tins or pouches)	
Commercial dry food (e.g. kibble or pellets)	
Commercially prepared raw diet	
Home-prepared uncooked/raw meat	
Home-prepared cooked meat	
Table scraps and leftovers	
I don't know what diet my puppy was on before I got it	
Other (please describe briefly)	

7. In which of these environments are you expecting to be regularly walking your puppy when they are old enough?

	Never	Sometimes	Often	Almost always
Roads and streets				
Parks				
Beaches				
Countryside without livestock animals				
Countryside with livestock animals				
Countryside with cattle				

Figure 2. The questionnaire provided to owners of locally recruited 12-week-old puppies. The questionnaire was completed and sent alongside a faecal sample collected from the puppy.

University of BRISTOL HSTAR

For office use only

Questionnaire 2

An investigation into carriage of antimicrobial resistant *E. coli* in young puppies

1. Today's date_____ Your puppy's name _____

2. Your name_____

3. Your puppy's age and/or date of birth_____

4. To the nearest week, at what age did your puppy start walking in public places? _____

5. Which types of food have you mostly fed your puppy on? Please tick all that apply

Commercial wet food (e.g. tins or pouches)	
Commercial dry food (e.g. kibble or pellets)	
Commercially prepared raw diet	
Home-prepared uncooked/raw meat	
Home-prepared cooked meat	
Table scraps and leftovers	
Other (please describe briefly)	

6. In which of these environments has your puppy been walked?

	Never	Sometimes	Often	Very often
Roads and streets				
Parks				
Beaches				
Countryside without livestock animals				
Countryside with livestock animals				
Countryside with cattle				

7. Has your puppy ever swum, paddled or played in any of the following?

	Never	Sometimes	Often	Very often
Salt water				
Lake water				
River water				
Pond water				
Other water source (please describe briefly)				

Please turn over

8. Has your puppy been unwell and therefore had antibiotics given or prescribed by your vet at any time? If so, please give brief details of the reason for antibiotic use and what was given.

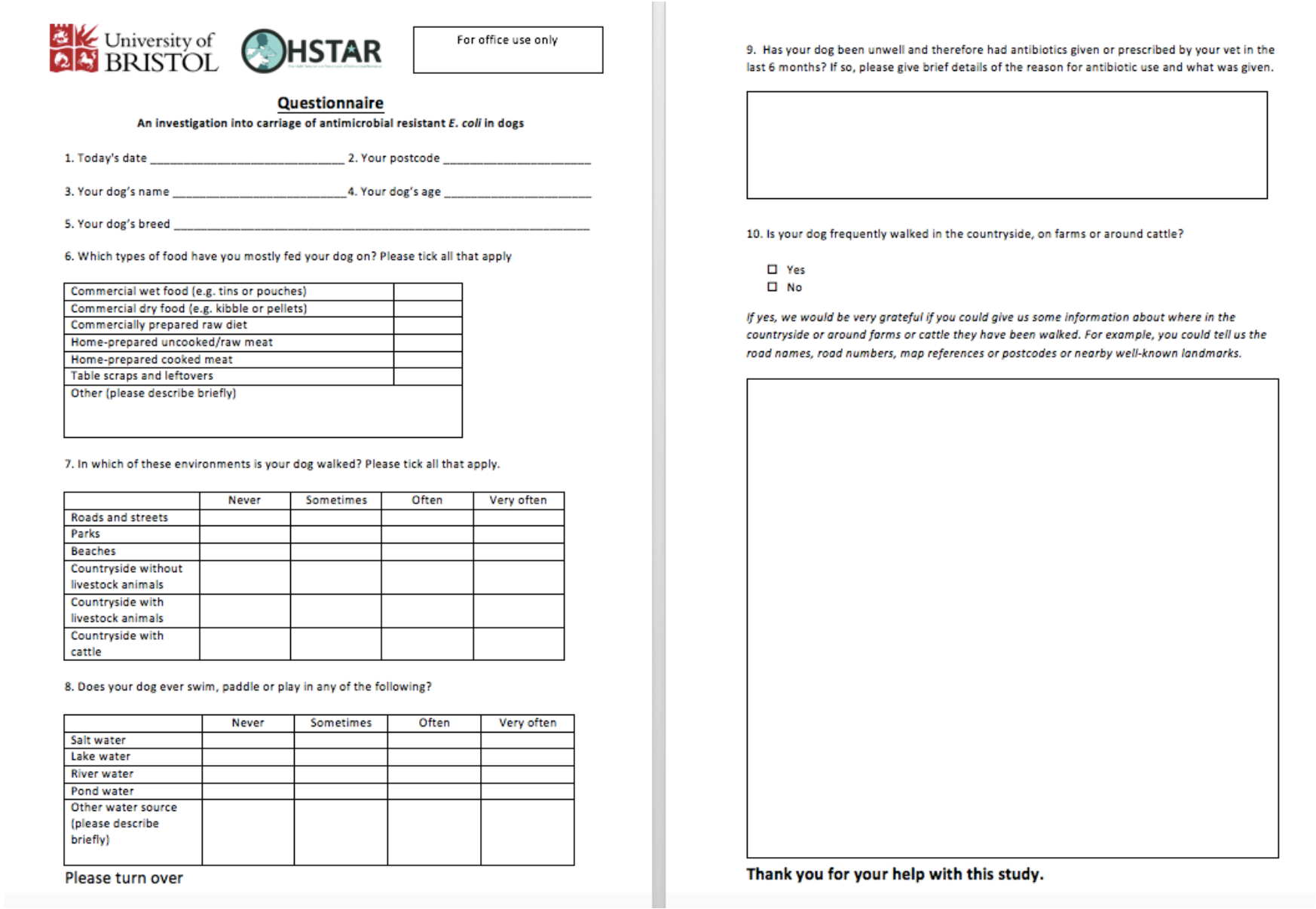
9. Has your puppy frequently been walked in the countryside, on farms or around cattle?

☐ Yes
☐ No

If yes, we would be very grateful if you could give us some information about where in the countryside or around farms or cattle they have been walked. For example, you could tell us the road names, road numbers, map references or postcodes or nearby well-known landmarks.

Thank you for your help with this study.

Figure 3. The second questionnaire provided to the locally recruited puppy owners when their puppy was 16 weeks old. The questionnaire was completed and sent alongside a faecal sample collected from the puppy.



University of BRISTOL HSTAR

For office use only

Questionnaire

An investigation into carriage of antimicrobial resistant *E. coli* in dogs

1. Today's date _____ 2. Your postcode _____

3. Your dog's name _____ 4. Your dog's age _____

5. Your dog's breed _____

6. Which types of food have you mostly fed your dog on? Please tick all that apply

Commercial wet food (e.g. tins or pouches)	
Commercial dry food (e.g. kibble or pellets)	
Commercially prepared raw diet	
Home-prepared uncooked/raw meat	
Home-prepared cooked meat	
Table scraps and leftovers	
Other (please describe briefly)	

7. In which of these environments is your dog walked? Please tick all that apply.

	Never	Sometimes	Often	Very often
Roads and streets				
Parks				
Beaches				
Countryside without livestock animals				
Countryside with livestock animals				
Countryside with cattle				

8. Does your dog ever swim, paddle or play in any of the following?

	Never	Sometimes	Often	Very often
Salt water				
Lake water				
River water				
Pond water				
Other water source (please describe briefly)				

Please turn over

9. Has your dog been unwell and therefore had antibiotics given or prescribed by your vet in the last 6 months? If so, please give brief details of the reason for antibiotic use and what was given.

10. Is your dog frequently walked in the countryside, on farms or around cattle?

☐ Yes
☐ No

If yes, we would be very grateful if you could give us some information about where in the countryside or around farms or cattle they have been walked. For example, you could tell us the road names, road numbers, map references or postcodes or nearby well-known landmarks.

Thank you for your help with this study.

Figure 4. The questionnaire provided to locally recruited adult dog owners. The questionnaire was completed and sent alongside a faecal sample collected from the dog.

Table 1. Univariable risk factor analyses using questionnaire data and AMR *E. coli* data for 16-week-old puppies recruited locally and via Generation Pup (excluding samples with a limit of detection issue where the *E. coli* count was less than 20 cfu; *n*=223). Odds ratio (95% confidence interval) *p*-value. *P*-value considered significant if below 0.05.

Risk factor	Resistant to any antibiotic (n=108)	Resistant to ciprofloxacin (n=26)	Resistant to tetracycline (n=81)	Resistant to amoxicillin (n=93)	Resistant to cephalexin (n=34)	Resistant to streptomycin (n=51)
Fed raw food	**3.98 (1.89 to 8.40) <0.001**	**12.42 (5.01to 30.78) <0.001**	**4.47 (2.21 to 9.05) <0.001**	**3.30 (1.64 to 6.63) 0.001**	1.97 (0.86 to 4.51) 0.11	**8.23 (3.95 to 17.15) <0.001**
Walked in town	0.65 (0.33 to 1.28) 0.21	3.06 (0.69 to 13.48) 0.14	0.63 (0.32 to 1.25) 0.18	0.74 (0.38 to 1.46) 0.39	0.88 (0.35 to 2.18) 0.78	0.69 (0.32 to 1.46) 0.33
Walked on farmland	1.02 (0.59 to 1.76) 0.95	1.63 (0.66 to 4.07) 0.29	1.04 (0.59 to 1.83) 0.90	1.06 (0.61 to 1.85) 0.83	1.05 (0.49 to 2.26) 0.89	1.33 (0.68 to 2.59) 0.40
Walked on beaches	1.17 (0.69 to 1.97) 0.57	**2.44 (1.04 to 5.74) 0.04**	1.55 (0.89 to 2.68) 0.12	1.00 (0.59 to 1.71) 0.99	1.38 (0.66 to 2.86) 0.39	0.94 (0.50 to 1.77) 0.86
Walked in countryside	1.45 (0.68 to 3.09) 0.34	2.16 (0.48 to 9.60) 0.31	**2.26 (0.93 to 5.49) 0.07**	1.23 (0.57 to 2.65) 0.60	1.87 (0.54 to 5.53) 0.33	1.71 (0.62 to 4.70) 0.30
Walked around cows	0.75 (0.44 to 1.30) 0.31	0.57 (0.23 to 1.43) 0.23	0.88 (0.50 to 1.55) 0.66	0.73 (0.42 to 1.27) 0.26	1.19 (0.57 to 2.50) 0.65	0.55 (0.28 to 1.09) 0.90

Swum/ paddled/ played in salt water	1.19 (0.66 to 2.14) 0.56	1.74 (0.74 to 4.08) 0.20	1.53 (0.84 to 2.78) 0.17	1.11 (0.61 to 2.01) 0.73	1.29 (0.59 to 2.84) 0.52	0.75 (0.36 to 1.55) 0.44
Swum/ paddled/ played in lake water	1.60 (0.73 to 3.54) 0.24	**3.72 (1.44 to 9.61) 0.007**	2.01 (0.95 to 4.56) 0.07	1.87 (0.85 to 4.11) 0.12	1.97 (0.77 to 5.05) 0.16	1.96 (0.85 to 4.55) 0.12
Swum/ paddled/ played in river water	1.09 (0.62 to 1.94) 0.76	1.57 (0.67 to 3.68) 0.30	1.10 (0.61 to 1.99) 0.75	1.14 (0.64 to 2.04) 0.66	0.57 (0.24 to 1.39) 0.22	0.99 (0.50 to 1.96) 0.97
Swum/ paddled/ played in pond water	1.77 (0.99 to 3.18) 0.06	1.94 (0.84 to 4.49) 0.12	**1.80 (1.00 to 3.25) 0.05**	2.01 (1.12 to 3.61) 0.02	1.40 (0.65 to 3.03) 0.39	1.29 (0.66 to 2.53) 0.46
Rolled in cowpat	4.83 (0.55 to 42.21) 0.15	1.34 (0.15 to 11.98) 0.79	3.13 (0.56 to 17.55) 0.20	6.35 (0.73 to 55.44) 0.10	**5.56 (1.06 to 28.98) 0.04**	**6.63 (1.17 to 37.53) 0.03**
Rolled in fox faeces	0.73 (0.19 to 2.81) 0.65	0.82 (0.10 to 6.87) 0.86	0.74 (0.18 to 3.07) 0.68	0.96 (0.25 to 3.70) 0.96	0.66 (0.08 to 5.19) 0.66	0.37 (0.04 to 3.04) 0.35
Displayed autocophrophagia behaviour in past seven days	**0.28 (0.09 to 0.90) 0.03**	---------	**0.19 (0.04 to 0.88) 0.03**	**0.25 (0.07 to 0.91) 0.04**	----------	

Table 2. Multivariable risk factor analyses using questionnaire data and AMR *E. coli* data for 16-week-old puppies recruited locally and via Generation Pup (excluding samples with a limit of detection issue where the *E. coli* count was less than 20 cfu; *n*=223). Odds ratio (95% confidence interval) *p*-value. *P*-value considered significant if below 0.05.

Risk factor	Resistant to any antibiotic (n=108)	Resistant to ciprofloxacin (n=26)	Resistant to tetracycline (n=81)	Resistant to amoxicillin (n=93)	Resistant to cephalexin (n=34)	Resistant to streptomycin (n=51)
Fed raw food	**3.88 (1.73 to 8.70) 0.001**	**11.32 (4.05 to 31.63) <0.001**	**4.18 (1.94 to 9.03) <0.001**	**3.14 (1.47 to 6.71) 0.003**	1.72 (0.69 to 4.28) 0.24	**7.84 (3.48 to 17.65) <0.001**
Walked in town	0.71 (0.34 to 1.46) 0.35	4.22 (0.86 to 20.82) 0.08	0.60 (0.29 to 1.28) 0.19	0.89 (0.43 to 1.81) 0.74	0.86 (0.33 to 2.24) 0.77	0.85 (0.36 to 2.00) 0.71
Walked on farmland	0.81 (0.37 to 1.77) 0.60	1.22 (0.34 to 4.40) 0.76	0.56 (0.25 to 1.27) 0.17	1.04 (0.48 to 2.28) 0.92	0.78 (0.28 to 2.18) 0.64	1.41 (0.56 to 3.58) 0.46
Walked on beaches	1.05 (0.48 to 2.31) 0.90	1.77 (0.47 to 6.68) 0.40	1.35 (0.59 to 3.10) 0.48	0.83 (0.37 to 1.86) 0.66	1.33 (0.47 to 3.76) 0.60	0.85 (0.31 to 2.32) 0.76
Walked in countryside	1.32 (0.52 to 3.34) 0.56	0.92 (0.15 to 5.79) 0.93	2.30 (0.80 to 6.61) 0.12	0.93 (0.36 to 2.39) 0.89	1.77 (0.42 to 7.37) 0.33	1.30 (0.62 to 4.70) 0.30
Walked around cows	0.81 (0.41 to 1.61) 0.55	0.47 (0.15 to 1.45) 0.19	1.02 (0.50 to 2.12) 0.92	0.73 (0.37 to 1.47) 0.38	1.38 (0.55 to 3.44) 0.49	0.47 (0.20 to 1.10) 0.08
Swum/ paddled/ played in salt water	1.06 (0.43 to 2.59) 0.90	0.95 (0.25 to 3.66) 0.94	1.19 (0.47 to 3.01) 0.71	1.06 (0.43 to 2.63) 0.90	1.07 (0.34 to 3.36) 0.90	0.69 (0.22 to 2.15) 0.52

Swum/ paddled/ played in lake water	0.85 (0.34 to 2.15) 0.74	1.28 (0.38 to 4.26) 0.69	1.03 (0.41 to 2.60) 0.95	1.11 (0.45 to 2.73) 0.81	1.58 (0.53 to 4.70) 0.41	1.01 (0.35 to 2.89) 0.99
Swum/ paddled/ played in river water	0.95 (0.48 to 1.90) 0.89	1.04 (0.33 to 3.26) 0.07	0.85 (0.41 to 1.75) 0.66	0.96 (0.48 to 1.92) 0.90	0.38 (0.14 to 1.05) 0.06	0.94 (0.39 to 2.25) 0.89
Swum/ paddled/ played in pond water	1.66 (0.83 to 3.33) 0.15	1.64 (0.54 to 4.98) 0.39	1.50 (0.73 to 3.06) 0.27	1.95 (0.98 to 3.90) 0.06	1.41 (0.57 to 3.50) 0.45	1.19 (0.51 to 2.75) 0.69

Table 3. Multivariable risk factor analyses using questionnaire data and AMR *E. coli* data for 16-week-old puppies recruited via Generation Pup (excluding samples with a limit of detection issue where the *E. coli* count was less than 20 cfu; *n*=182). Odds ratio (95% confidence interval) *p*-value. *P*-value considered significant if below 0.05.

Risk factor	Resistant to any antibiotic (n=94)	Resistant to ciprofloxacin (n=24)	Resistant to tetracycline (n=72)	Resistant to amoxicillin (n=82)	Resistant to cephalexin (n=30)	Resistant to streptomycin (n=45)
Fed raw food	**3.32 (1.39 to 7.95) 0.007**	**9.82 (3.26 to 29.54) <0.001**	**3.71 (1.58 to 8.67) 0.003**	**2.33 (1.03 to 5.27) 0.04**	1.16 (0.43 to 3.14) 0.77	**6.93 (2.83 to 16.98) <0.001**
Walked in town	0.79 (0.36 to 1.71) 0.54	4.59 (0.90 to 23.30) 0.07	0.61 (0.27 to 1.37) 0.23	1.06 (0.49 to 2.30) 0.88	1.06 (0.39 to 2.88) 0.91	1.00 (0.40 to 2.53) 1.00
Walked on farmland	0.88 (0.37 to 2.11) 0.77	1.42 (0.37 to 5.43) 0.61	0.55 (0.22 to 1.35) 0.19	1.18 (0.49 to 2.80) 0.72	1.15 (0.37 to 3.53) 0.81	2.07 (0.77 to 5.56) 0.15
Walked on beaches	0.72 (0.28 to 1.86) 0.50	1.40 (0.31 to 6.41) 0.66	0.81 (0.30 to 2.21) 0.68	0.68 (0.26 to 1.76) 0.43	1.83 (0.53 to 6.33) 0.34	0.66 (0.19 to 2.30) 0.52
Walked in countryside	1.24 (0.37 to 4.15) 0.72	1.07 (0.10 to 11.68) 0.95	**4.65 (1.05 to 20.63) 0.04**	1.03 (0.31 to 3.46) 0.96	0.65 (0.14 to 3.04) 0.58	2.55 (0.44 to 14.86) 0.30
Walked around cows	0.71 (0.32 to 1.57) 0.40	0.40 (0.28 to 5.52) 0.78	0.88 (0.38 to 2.03) 0.76	0.57 (0.25 to 1.26) 0.16	0.69 (0.23 to 2.06) 0.51	**0.23 (0.08 to 0.65) 0.006**
Swum/ paddled/ played in salt water	1.68 (0.59 to 4.79) 0.33	0.98 (0.27 to 3.51) 0.97	2.12 (0.71 to 6.31) 0.18	1.30 (0.46 to 3.70) 0.62	1.25 (0.35 to 4.52) 0.73	0.92 (0.24 to 3.55) 0.91

Swum/ paddled/ played in lake water	0.70 (0.25 to 1.95) 0.50	1.38 (0.42 to 4.50) 0.59	0.74 (0.26 to 2.07) 0.56	1.94 (0.35 to 2.54) 0.90	1.31 (0.40 to 4.29) 0.65	0.65 (0.19 to 2.14) 0.48
Swum/ paddled/ played in river water	0.86 (0.40 to 1.84) 0.70	1.64 (0.42 to 4.50) 0.59	0.85 (0.38 to 1.89) 0.70	0.75 (0.35 to 1.63) 0.47	0.36 (0.12 to 1.10) 0.07	0.81 (0.30 to 2.16) 0.67
Swum/ paddled/ played in pond water	1.66 (0.77 to 3.56) 0.19	1.64 (0.53 to 5.08) 0.39	1.69 (0.77 to 3.72) 0.19	1.91 (0.89 to 4.10) 0.10	1.53 (0.58 to 4.01) 0.39	1.07 (0.43 to 2.65) 0.88
Rolled in cow pats	3.84 (0.36 to 40.64) 0.26	0.95 (0.05 to 16.92) 0.97	1.91 (0.24 to 14.99) 0.54	6.06 (0.59 to 62.31) 0.13	**9.50 (1.27 to 71.05) 0.03**	**12.16 (1.49 to 99.49) 0.02**
Rolled in fox faeces	0.60 (0.14 to 2.61) 0.50	0.33 (0.02 to 4.59) 0.41	0.61 (0.13 to 2.87) 0.53	0.77 (0.18 to 3.36) 0.73	0.73 (0.08 to 6.76) 0.78	0.24 (0.02 to 2.37) 0.22
Displayed autocoprophagic behaviour in past seven days	**0.21 (0.05 to 0.82) 0.02**	---	**0.09 (0.01 to 0.76) 0.03**	**0.16 (0.03 to 0.79) 0.03**	---	---

Table 4. Univariable risk factor analyses using questionnaire data and AMR E. coli data for 12-week-old puppies (excluding samples with a limit of detection issue where the *E. coli* count was less than 20 cfu*; n*=64). Odds ratio (95% confidence interval) *p*-value. *P*-value considered significant if below 0.05. The questionnaire answers were of where the owner intended to walk their puppy when the puppy was old enough to walk in public places.

Risk factor	Resistant to any antibiotic (n=33)	Resistant to ciprofloxacin (n=5)	Resistant to tetracycline (n=13)	Resistant to amoxicillin (n=32)	Resistant to cephalexin (n=8)	Resistant to streptomycin (n=12)
Fed raw food	2.90 (0.28 to 29.51) 0.37	4.50 (0.38 to 53.77) 0.24	1.28 (0.12 to 13.41) 0.84	3.00 (0.29 to 30.56) 0.35	2.48 (0.23 to 27.32) 0.46	1.42 (0.13 to 15.03) 0.77
Walked on roads/streets	NT	NT	NT	NT	NT	NT
Walked in parks	0.69 (0.11 to 4.45) 0.70	0.31 (0.03 to 3.45) 0.34	0.98 (0.10 to 9.64) 0.99	0.67 (0.10 to 4.30) 0.67	0.18 (0.02 to 1.28) 0.09	0.33 (0.05 to 2.21) 0.25
Walked on beaches	0.52 (0.09 to 3.06) 0.47	0.38 (0.04 to 4.14) 0.43	NT	0.50 (0.08 to 2.95) 0.44	0.24 (0.04 to 1.57) 0.14	NT
Walked in countryside (no livestock)	1.54 (0.31 to 7.52) 0.60	NT	1.67 (0.18 to 15.29) 0.65	1.49 (0.30 to 7.28) 0.62	NT	NT

Walked in countryside (with livestock)	2.80 (0.83 to 9.46) 0.10	0.44 (0.07 to 2.94) 0.40	1.99 (0.39 to 10.18) 0.41	2.70 (0.80 to 9.14) 0.11	2.39 (0.27 to 21.17) 0.43	0.95 (0.22 to 4.08) 0.94
Walked in countryside (with cattle)	2.25 (0.70 to 7.22) 0.17	0.49 (0.07 to 3.23) 0.46	2.20 (0.43 to 11.22) 0.34	2.17 (0.67 to 6.97) 0.19	2.63 (0.30 to 23.17) 0.39	1.05 (0.25 to 4.50) 0.94

Table 5. Multivariable risk factor analyses using questionnaire data and AMR E. coli data for 12-week-old puppies (excluding samples with a limit of detection issue where the *E. coli* count was less than 20 cfu*; n*=64). Odds ratio (95% confidence interval) *p*-value. *P*-value considered significant if below 0.05. The questionnaire answers were of where the owner intended to walk their puppy when the puppy was old enough to walk in public places.

Risk factor	Resistant to any antibiotic (n=33)	Resistant to ciprofloxacin (n=5)	Resistant to tetracycline (n=13)	Resistant to amoxicillin (n=32)	Resistant to cephalexin (n=8)	Resistant to streptomycin (n=12)
Fed raw food	1.70 (0.14 to 20.37) 0.68	25.81 (0.94 to 710.72) 0.06	NT	1.79 (0.15 to 21.49) 0.65	6.13 (0.41 to 91.57) 0.19	2.25 (0.17 to 29.06) 0.53
Walked on roads/streets	NT	NT	NT	NT	NT	NT
Walked in parks	0.67 (0.10 to 4.58) 0.68	0.06 (0.002 to 1.45) 0.08	0.96 (0.09 to 10.57) 0.97	0.65 (0.10 to 4.41) 0.66	**0.08 (0.008 to 0.83) 0.03**	0.22 (0.03 to 1.91) 0.17
Walked on beaches	0.20 (0.02 to 2.22) 0.19	0.08 (0.004 to 1.89) 0.12	NT	0.20 (0.02 to 2.16) 0.19	**0.03 (0.002 to 0.51) 0.02**	NT
Walked in countryside (no livestock)	4.40 (0.36 to 53.65) 0.25	NT	NT	4.34 (0.36 to 53.11) 0.25	NT	NT
Walked in countryside (with livestock)	NT	NT	NT	NT	NT	NT

Walked in countryside (with cattle)	NT	NT	NT	NT	NT	NT

NT = not tested due to collinearity (this is due to small sample size)

Table 6. Univariable risk factor analyses using questionnaire data and AMR E. coli data for adult dogs recruited locally (excluding samples with a limit of detection issue where *the E. coli* count was less than 20 cfu; *n*=14). Odds ratio (95% confidence interval) *p*-value. *P*-value considered significant if below 0.05.

Risk factor	Resistant to any antibiotic (n=5)	Resistant to ciprofloxacin (n=2)	Resistant to tetracycline (n=4)	Resistant to amoxicillin (n=3)	Resistant to cephalexin (n=1)	Resistant to streptomycin (n=3)
Walked on roads/ streets	0.08 (0.01 to 1.29) 0.08	0.33 (0.02 to 7.14) 0.48	0.25 (0.02 to 3.04) 0.28	0.11 (0.01 to 1.92) 0.13	NT	0.75 (0.05 to 11.65) 0.84
Walked in parks	0.83 (0.09 to 7.68) 0.87	NT	1.50 (0.15 to 15.46) 0.73	3.50 (0.24 to 51.90) 0.36	NT	3.50 (0.24 to 51.90) 0.36
Walked on beaches	0.21 (0.01 to 3.37) 0.27	0.22 (0.01 to 5.28) 0.35	0.13 (0.01 to 2.18) 0.15	0.50 (0.03 to 8.71) 0.63	NT	0.50 (0.03 to 8.71) 0.63
Walked in countryside (no livestock)	NT	NT	NT	NT	NT	NT
Walked in countryside (with livestock)	0.50 (0.02 to 10.25) 0.65	NT	NT	0.20 (0.01 to 4.72) 0.32	NT	NT
Walked in countryside (with cattle)	0.50 (0.02 to 10.25) 0.65	NT	NT	0.20 (0.01 to 4.72) 0.32	NT	NT

Swum/ paddled/ played in salt water	0.83 (0.09 to 7.68) 0.87	1.4 (0.07 to 28.12) 0.83	1.50 (0.15 to 15.46) 0.73	0.60 (0.04 to 8.73) 0.71	NT	0.60 (0.04 to 8.73) 0.71
Swum/ paddled/ played in lake water	1.33 (0.14 to 12.82) 0.80	2.00 (0.10 to 41.00) 0.65	2.33 (0.22 to 25.24) 0.49	0.88 (0.06 to 12.97) 0.92	NT	0.88 (0.06 to 12.98) 0.92
Swum/ paddled/ played in river water	3.20 (0.25 to 41.21) 0.37	NT	NT	1.14 (0.08 to 16.95) 0.92	NT	NT
Swum/ paddled/ played in pond water	1.88 (0.20 to 17.27) 0.58	NT	4.50 (0.34 to 60.15) 0.26	2.40 (0.16 to 34.93) 0.52	NT	NT
Recently received antibiotics	NT	NT	NT	NT	NT	NT
Walked frequently around cattle	0.19 (0.01 to 2.91) 0.23	0.20 (0.01 to 4.72) 0.32	0.75 (0.05 to 11.65) 0.84	0.05 (0.002 to 1.18) 0.06	NT	0.44 (0.03 to 7.67) 0.58

NT = not tested due to collinearity (this is due to small sample size)